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The 'Selenastrum capricornutum' Printz Algal Assay Bottle Test: Experimental Design, Application, and Data Interpretation Protocol

Corvallis Environmental Research Lab, OR

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Corvallis, Oregon 97330

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July 1978

THE *SELENASTRUM CAPRICORNUTUM* PRINTZ ALGAL ASSAY BOTTLE TEST

Experimental Design, Application, and Data Interpretation Protocol

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This report supercedes the Algal Assay Procedure: Bottle test (USEPA 1971).

16. ABSTRACT
This document is the product of intensive research to improve and expand the understanding of results obtained from the Algal Assay Procedure: Bottle Test (USEPA 1971) to enable investigators to define the stimulatory and/or inhibitory interaction(s) of municipal, industrial and agri cultural wastes upon algal productivity in natural waters.

This research was designed to determine:

1. The impact of nutrients and/or changes in their loading upon algal productivity;
2. Whether the growth response of Selenastrum capricornutum reflects the response of indigenous species;
3. The feasibility of the assay test protocol to evaluate heavy metals;
4. The capability of the assay to define the effect(s) of complex wastes; and
5. If the assay information can be applied to define and assist in the management of real-world situation.

As a result of these research efforts the Selenastrum capricornutum Printz Algal Assay Bottle Test: Experimental Design, Application and Data Interpretation Guide is offered now for wider application in both eutrophication and toxicity problem areas.

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by

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DISCLAIMER

This report has been reviewed by the Corvallis Environmental Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

Effective regulatory and enforcement actions by the Environmental Protection Agency would be virtually impossible without sound scientific data on pollutants and their impact on environmental stability and human health. Responsibility for building this data base has been assigned to EPA's Office of Research and Development and its 15 major field installations, one of which is the Corvallis Environmental Research Laboratory (CERL).

The primary mission of the Corvallis Laboratory is research on the effects of environmental pollutants on terrestrial, freshwater, and marine ecosystems; the behavior, effects and control of pollutants in lake systems; and the development of predictive models on the movement of pollutants in the biosphere.

This report reflects the latest research findings of the continued refinement, evaluation and application of algal assays to study the effects of pollutants upon algal productivity in natural waters. This test protocol can be used to evaluate nutrients, heavy metals, new product formulations and complex wastes.

A. F. Bartsch
Director, CERL

PREFACE

This document is the product of intensive research to improve and expand the understanding of results obtained from the Algal Assay Procedure: Bottle Test (USEPA, 1971) to enable investigators to define the stimulatory and/or inhibitory interaction(s) of municipal, industrial and agricultural wastes upon algal productivity in natural waters.

This research was designed to determine:

(1) The impact of nutrients and/or changes in their loading upon algal productivity;

(2) Whether the growth response of _Selenastrum_ _capricornutum_ reflects the response of indigenous species;

(3) The feasibility of the assay test protocol to evaluate heavy metals;

(4) The capability of the assay to define the effect(s) of complex wastes; and

(5) If the assay information can be applied to define and assist in the management of real-world situation.

As a result of these research efforts the _Selenastrum_ _capricornutum_ Printz Algal Assay Bottle Test: Experimental Design, Application and Data Interpretation Guide is offered now for wider application in both eutrophication and toxicity problem areas. This point in progress has been attained through the dedication and continuing energies of Mr. Miller, Mr. Greene and Mr. Shiroyama. To them goes much credit for the effective way in which the research effort moved continuously and effectively toward the refinement and application of the Algal Assay Procedure: Bottle Test.

The research could not have been completed without the efforts of Ethan Bergman, Kurt Putnam, Ellen Merwin, Mike Long and Amy Leischman and others who provided laboratory support on various research projects.

Special appreciation is also extended to Amy Leischman and Mike Long for editing the bibliography and compiling the mailing list. The untiring, cheerful support of Nancy Cruse, who typed this document and suffered through its many revisions is also greatly appreciated. Acknowledgement is also given to Howard Mercier for providing the FORTRAN data reduction program.

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1.0 Introduction

Algae are natural inhabitants of waters and are an extremely important group of plant organisms. Through their photosynthetic activity they help to provide the oxygen necessary for the survival of animal species found in the aquatic environment. Algae contribute to the self purification of streams, lakes and estuaries, and also serve as the basis of the food chain within the aquatic ecosystem. However, when nutrients and sunlight are plentiful they are capable of rapid growth and multiplication. This often results in serious water quality problems. In the absence of sunlight they deplete the oxygen levels through their respiratory activity. Heavy growths, or "algal blooms" often cause tastes and odors in drinking water supplies. Some algal species produce metabolic products that are toxic and have been implicated in the death of livestock, waterfowl and fish. Because of the widespread interest in algae, strong emphasis has been placed upon having a standard and reproducible method for determining the potential of waters, sewage and industrial effluents, and various compounds to support, accelerate or inhibit algal growth.

The significance of measuring the algal growth potential of water is that a differentiation can be made between the nutrients that are in the sample (as determined by chemical analysis) and the nutrient forms that are actually available for algal growth. The addition of a given nutrient(s) to a sample can give an indication of which nutrient(s) is limiting for algal growth. Also, if algal growth remains limited when nutrients are in sufficient supply and the physical conditions for growth exist, the presence of a toxicant is indicated (Miller, Maloney and Greene, 1974; Greene *et al*., 1975; Payne, 1976; Gerhold, 1976; Greene *et al*., 1976).

The interpretation of actual algal assay results depends on the reliability of the test procedure. To be effective an assay experiment should be designed to include built-in checks and balances of known growth responses, such as to standard additions of nitrogen and phosphorus singularly or in combination. The amount of growth response of the test organism can be used to verify both chemical analyses for nitrogen and phosphorus and the precision and accuracy of the assay response. Failure of a test water to attain the predicted yield or nutrient limitation status can usually be attributed to one or more of the following causes: (1) absence of other growth requiring nutrients; (2) the presence of toxicants; or (3) unreliable chemical analysis for Ortho-P and total soluble inorganic nitrogen (NO_2 + NO_3 + NH_3-N = TSIN).

The use of standard laboratory algal test species, grown under specific environmental culture conditions, is essential to the understanding of the complex interaction of nutrient and/or inhibitor laden wastes upon aquatic productivity. Odum (1971) discussed the use of unialgal cultures as being prerequisite to defining the growth effect of each nutrient in relationship to the combined effects of all other factors within the entire complex of conditions. Detection of algal growth reactions, whether inhibitory or stimulatory, becomes more precise as detailed background information accumulates on the physiology of a given test species.

When comparing algal growth potentials from a number of widely different water sources there are advantages in using the same species of algae for all waters. The alga to be used must be readily available and its growth must be able to be measured easily and accurately. It must also respond to growth substances uniformly. Some algae are capable of concentrating certain nutrients in excess of their normal metabolic requirements. Therefore, this factor must be taken into account in selecting the culture medium and in determining the type and amount of algae to use. If algae are cultured in a relatively dilute medium, as recommended in the "Algal Assay Procedure: Bottle Test" (USEPA, 1971), the amount of growth in subsequent testwaters resulting from nutrient carryover is minimized. Experiments with the green alga, *Chlorella pyrenoidosa*, grown in this relatively dilute medium, disclosed no significant further growth in media lacking nitrogen or phosphorus. This was true even when these algae were transferred from the initial medium over a wide range of inoculum sizes (Fitzgerald, 1972).

Isolation of a single indigenous algal species, even if that alga were dominant at the time of sampling, does not mean that when grown in laboratory culture it is more indicative of natural conditions than a laboratory species. The use of an indigenous algal species isolated for use as a specific laboratory test organism is not recommended. The dynamics of natural phytoplankton blooms, in which the dominant algal species changes throughout the growth season, makes it quite certain that even if the indigenous algal isolate were dominant at the time of collection, many other species will dominate the standing crop as the season progresses.

The presence of indigenous algae in a water sample suggests that they are the most fit to survive in the environment from which the sample was taken. Under adequate light and temperature conditions the indigenous algae should produce biomass until growth is limited by some essential nutrient or inhibitor. If the indigenous algae are limited from further growth by an essential nutrient, the laboratory test alga cultured in a non-competitive environment and responding to the same limiting nutrient will produce parallel maximum growth yield responses.

Generally, indigenous phytoplankton bioassays are not necessary unless there is strong evidence of the presence of persistent sub-lethal toxicants to which indigenous populations might have developed tolerance (Greene *et al*., 1978).

The extensive design, evaluation and application of algal assay research, centered around the use of *Selenastrum capricornutum* as the dominant test alga, has demonstrated the ability of unialgal assays to identify and assist in the management of major water quality problem areas. This document is the result of extensive research using the "Algal Assay Procedure: Bottle test," developed by the Environmental Protection Agency, for assaying algal growth potential in natural water samples (USEPA, 1971). It is this work on which the following test is based.

2.0 PRINCIPLE

This assay procedure is based upon *a modification of Liebig's Law of the minimum* which states that "maximum yield is proportional to the amount of a nutrient or combination of nutrients which are present and biologically available in minimal quantity in respect to the growth requirements of the organisms." As stated by Liebig, his law applies to a single nutrient limiting growth at any one time. This concept has been documented for the critical nitrogen and phosphorus requirements for optimum growth of *S. capricornutum* in both culture medium and natural waters providing other essential elements are *present in excess* (Shiroyama, Miller and Greene, 1975). However, the concept of a single limiting nutrient is not infallible. More than one nutrient can simultaneously limit growth. For example: the interaction of nitrogen and phosphorus can regulate maximum yield of *S. capricornutum* as the critical ratio of these elements approaches 11:1. Algal growth can often be stimulated in test waters containing this ratio of N:P by the combined addition of N and P spikes. These growth responses support the current modification of Liebig's Law which is considered valid for the interpretation of nutrient limitation obtained under conditions specified in this document.

The test in its present form is intended primarily for use in the following general situations:

1. Assessment of a receiving water to determine its nutrient status and sensitivity to changes in N and P loading.
2. Evaluation of materials and products to determine their potential stimulatory or inhibitory effects on algal growth in receiving waters.
3. Assessment of effects of complex wastes originating from industrial, municipal, and agricultural point or non-point sources to define their impact upon receiving waters.

The bottle test consists of three steps: (1) selection and measurement of biomass parameters during the assay (for example, biomass indicators such as dry weight); (2) presentation and statistical evaluation of the measurements made during the assay; and (3) interpretation of the results with respect to the specific problem being investigated. It is intended that the test be used: (1) to identify algal growth-limiting constituents; (2) to determine biologically the availability of algal growth-limiting nutrients; and (3) to quantify the biological response to changes in concentrations of algal growth-limiting constituents. These measurements are made by adding a selected test alga to the test water and determining algal growth (as dry weight) at appropriate intervals.

The test also may be used to determine whether or not complex wastes, inorganic or organic compounds, or receiving waters are inhibitory to algae. Caution should be observed in interpreting results where there is little or no growth response in samples when sufficient nutrients appear to be or are, in fact, present. The presence of toxicants can inhibit or prevent algal growth even when nutrients are not growth-limiting.

It should be pointed out that test flasks are normally incubated to facilitate free gas exchange at the air-water interface. Therefore, carbon dioxide is rarely growth-limiting except in cases where maximum yield exceeds 200 mg dry weight l^{-1}. Because of this design feature, the test as outlined cannot be used to define growth limitations of carbon in the test water. The test can be modified to obtain such information.

2.1 Growth response--Maximum standing crop (MSC) is proportional to the initial amount of limiting nutrient available providing other factors are not growth regulating. All comparative growth responses should be analyzed statistically and significant levels of the differences should be reported.

A statistical coefficient of variance analysis of the MSC replication obtained in 685 test waters (each consisting of 3 replicate flasks) for yields ranging between 0.01 and 130.00 mg dry wt l^{-1} are shown in Table 1.

TABLE 1
STATISTICAL COEFFICIENT OF VARIANCE ANALYSIS OF THE STANDING CROP (MSC) REPLICATION

No Samples	MSC	% Coefficient of Variance
66	0.01 - 0.09	47.8
80	0.10 - 0.99	45.4
26	1.00 - 1.99	27.1
40	2.00 - 2.99	26.4
27	3.00 - 3.99	19.6
27	4.00 - 4.99	17.7
29	5.00 - 5.99	17.8
31	6.00 - 6.99	14.4
22	7.00 - 7.99	12.5
25	8.00 - 8.99	13.8
14	9.00 - 9.99	12.5
86	10.00 - 14.99	11.8
39	15.00 - 19.99	11.6
20	20.00 - 24.99	9.0
11	25.00 - 29.99	10.2
2	30.00 - 34.99	3.2
7	35.00 - 39.99	8.5
7	40.00 - 49.99	7.2
13	50.00 - 59.99	7.5
33	60.00 - 69.99	6.3
32	70.00 - 79.99	6.8
11	80.00 - 89.99	8.2
17	90.00 - 99.99	8.1
14	100.00 - 109.99	8.7
2	110.00 - 119.99	9.0
4	120.00 - 130.00	7.8

The coefficient of variance decreases as the MSC increases. The higher values corresponding to MSC < 1.00 mg dry wt l^{-1} and the lower percent variance for yields > 10.00 mg dry wt l^{-1}. The following percent variance guidelines can be used to ascertain whether the differences obtained in MSC between replicate flasks and/or nutrient additions are statistically significant:

± 50% for MSC < 1.00 mg dry wt l^{-1}

± 30% for MSC > 1.00 but < 3.00 mg dry wt l^{-1}

± 20% for MSC > 3.00 but < 10.00 mg dry wt l^{-1}

± 10% for MSC > 10.00 mg dry wt l^{-1}

3.0 PLANNING OF ALGAL ASSAYS

The specific experimental design of each algal assay is dictated by the actual situation. It is extremely important that all pertinent environmental factors be considered in the planning of a given assay to insure that valid results and conclusions are obtained.

Resource availability (manpower, equipment, and dollars) often dictate the degree of sophistication of the assay. Therefore, the following specifics must be considered as an absolute minimum by each investigator who plans to conduct algal assays for the purposes listed above (see 2.0).

3.1 Selection of test waters--Water quality may vary greatly with time and with location in lakes, impoundments and streams. Sampling programs must be established so that meaningful data will be obtained.

3.11 Spatial variations--In a thermally stratified lake or impoundment, only depth integrated euphotic zone composite samples need be collected. In most cases, the euphotic zone is described as the depth to which at least 1% of the surface light is available. Euphotic depths greater than 8 meters should be subsampled at least at the surface and at each 3-meter depth interval. Likewise, euphotic zones less than 8 meters should be sampled at least at the surface and 2-meter intervals. Each equal volume depth sample must be composited in a suitable nonmetallic container and upon thorough mixing is subsampled for algal assay and chemical and biological analysis--including algal identification.

The use of transect lines are helpful in sampling. Samples from a transect can be taken from predetermined euphotic zones. Representative river samples can be identified by specific conductance measurements which show the homogeneity of the sampling transect. In rivers and streams useful information may be obtained by taking samples upstream and downstream from suspected pollutant sources or confluent tributaries.

New products should be evaluated for their stimulatory and/or inhibitory effect upon algal growth before being discharged into receiving waters. When new products or materials are evaluated, samples of natural waters from geographically different areas having a range of representative water quality (such as alkalinity, hardness, pH, and ionic strength) must be investigated.

3.12 Temporal variations--The nutrient content of natural and waste waters often varies greatly with time. The variation may not only be seasonal, but hourly. The effects of these variations in lakes and in impoundments must be considered and can be minimized when sampled in accordance with section 3.11.

3.2 Sample collection, transport, preparation and storage.

3.21 Collection--Use non-metallic water sampler and autoclavable storage containers (such as linear polyethylene, polypropylene, or polycarbonate). Containers should not be re-used when toxic or nutrient contamination is suspected.

3.22 Transport conditions--Leave a minimum of air space in the sample container, keep in the dark and packed in ice. (Taping the bottle cap helps to insure against leakage.)

3.23 Preparation--In order to use a unialgal test species the indigenous algae in the sample must be "removed" before assaying. This removal requires destruction and separation of the indigenous algae. Autoclaving followed by filtration is recommended when it is desired to determine the amount of algal biomass that can be grown from all nutrients in the water, including those contained in filterable organisms and other particulate matter, which can be solubilized by autoclaving. The sample should be autoclaved at 1.1 kg cm^2 (15 psi) at 121°C (250°F). The period of autoclaving will depend on the sample volume, e.g., 30 minutes or 10 minutes per liter, whichever is longer. After autoclaving and cooling, the sample should be equilibrated by bubbling with a 1% carbon dioxide and air mixture to restore the carbon dioxide lost during autoclaving and to lower the pH to its original level (it will generally rise on autoclaving). In some instances, waters with total hardness greater than 150 mg l^{-1} will lose calcium and phosphorus upon autoclaving. This precipitate may be resistant to resolubilization by addition of carbon dioxide and air. In waters containing high levels of hardness and alkalinity the pH may not increase upon autoclaving. It is recommended that 1% CO_2 and air mixture be bubbled through the sample for at least 2 minutes per liter. If an electronic particle counter is to be used for all counting, the carbon dioxide equilibrated sample must be passed through a 0.45μm membrane filter.

Autoclaving followed by filtration is the recommended pretreatment for nutrient limitation and heavy metal toxicity studies; however, its use in studies of complex wastes and organic compounds may alter the chemical structure and bias the assay response. Presently, filtration (0.45 μm) is the only recommended pretreatment prior to the assay of organic compounds and complex wastes. Filtration is essential to eliminate unwanted biological contaminants which would invalidate the growth response of the test organism.

3.24 Storage--Although changes can occur in pretreated water samples during storage, regardless of storage conditions, the extent or chemistry of these changes is not well defined. Attempts should be made to minimize the effect of storage by keeping samples cooled at 4°C in the dark, using proper containers, and avoiding air spaces over the sample.

4.0 APPARATUS

4.1 Sampling and sample preparation.

4.11 Water sampler--Non-metallic

4.12 Sample bottles--Autoclavable (such as polypropylene, linear polyethylene or polycarbonate).

4.13 Membrane filter apparatus--For use with 47 or 142 mm filter pads and 0.45µm porosity filters. To reduce filtration time, the larger membrane (142mm) filtration unit is recommended.

4.14 Autoclave or pressure cooker--Capable of producing 1.1 kg cm^2 (15 psi) at 121°C (250° F).

4.2 Culturing and incubation.

4.21 Culture vessels--Erlenmeyer flasks of good quality borosilicate glass such as Pyrex or Kimax. When trace nutrients are being studied, special glassware such as Vycor, polycarbonate, or coated glassware can be used.

The flask size is not critical but, due to carbon dioxide limitation, the sample to volume ratios are. The recommended sample to volume ratios are:

25 ml sample in 125 ml flask
50 ml sample in 250 ml flask
100 ml sample in 500 ml flask

These twenty percent sample to volume ratios are for flasks which are shaken by hand once daily. Maximum permissible sample to volume ratios in continuously shaken (100 rpm) flasks should not exceed 50%.

4.22 Shaker table--Capable of 100 revolutions per minute (Figures 1 and 1a). A schematic of the 500 ml flask platform is shown in appendix 11.2.

4.23 Culture closures--Foam plugs must be used to permit good gas exchange and prevent contamination. Each laboratory must determine for each batch of closures purchased whether that batch has any significant effect on the maximum standing crop.

4.24 Constant temperature room or equivalent incubator--Capable of providing temperature control at 24 ± 2°C (Figure 2).

4.25 Illumination--"Cool-White" fluorescent lighting to provide 4304 lumens (400 ± 10% ft-c) measured adjacent to the flask at the liquid level (Figure 3).

4.26 Light meter--Several types are acceptable, but the meter must be calibrated against a standard light source or light meter. Commercial laboratories can perform such standardizations by comparison with calibrated lamps supplied by the National Bureau of Standards. Standardization should include both intensity and color temperature comparisons if the light meter is designed for a color temperature different from the color-correlated temperature (4200°K) of the "Cool-White" fluorescent light source specified in the assay procedure.

4.27 pH meter--Scale of 0-14 pH units with accuracy of ± 0.1 pH unit.

4.3 Bioassessment evaluation

4.31 Electronic particle counter with mean cell volume computer (MCV).

4.32 Fluorometer--Suitable for measurement of chlorophyll a (see 8.53).

4.33 Microscope--General purpose.

4.34 Microscope illuminator--Good quality general purpose.

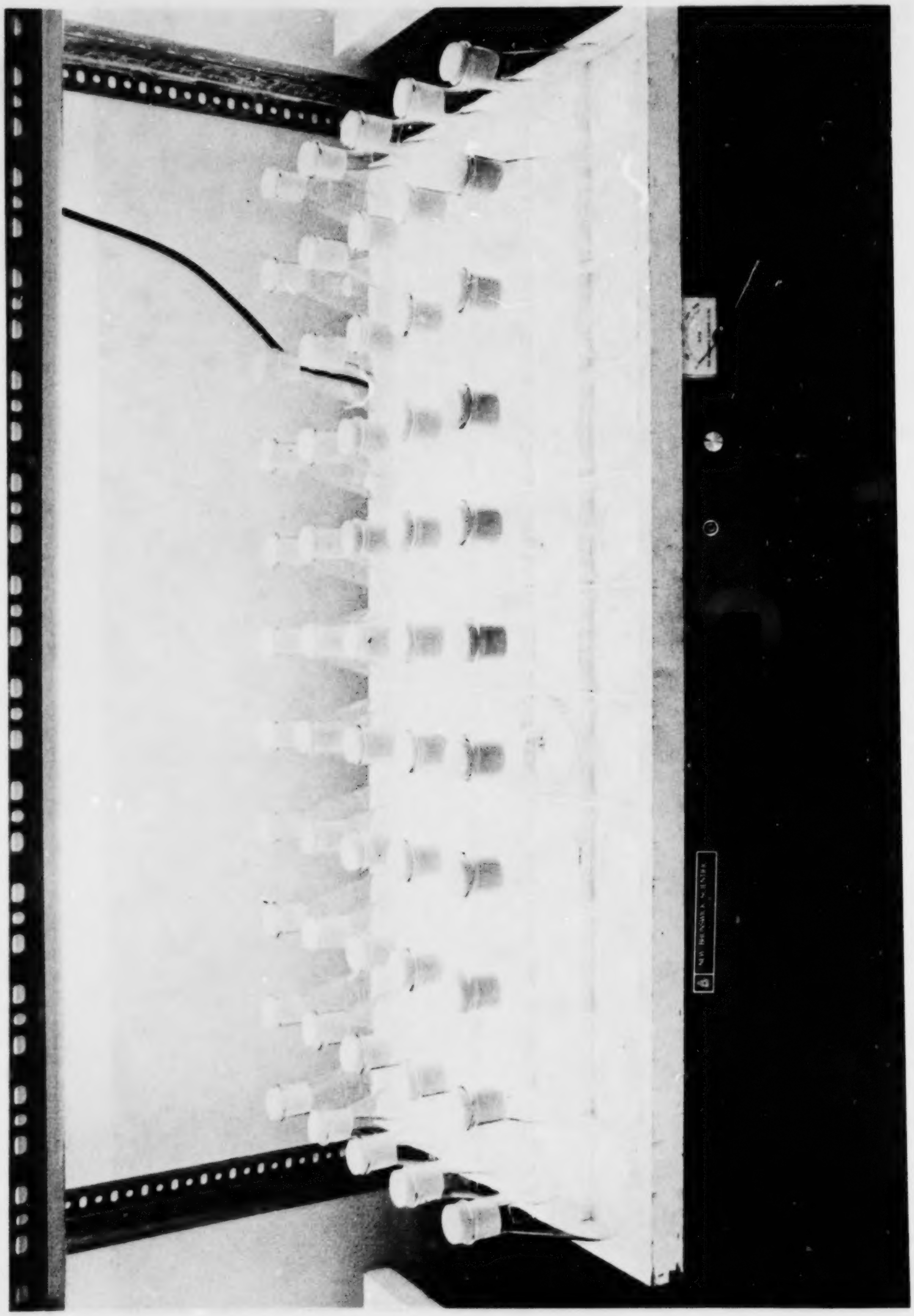

Figure 1. Shaker platform with 500 ml Erlenmeyer flasks.

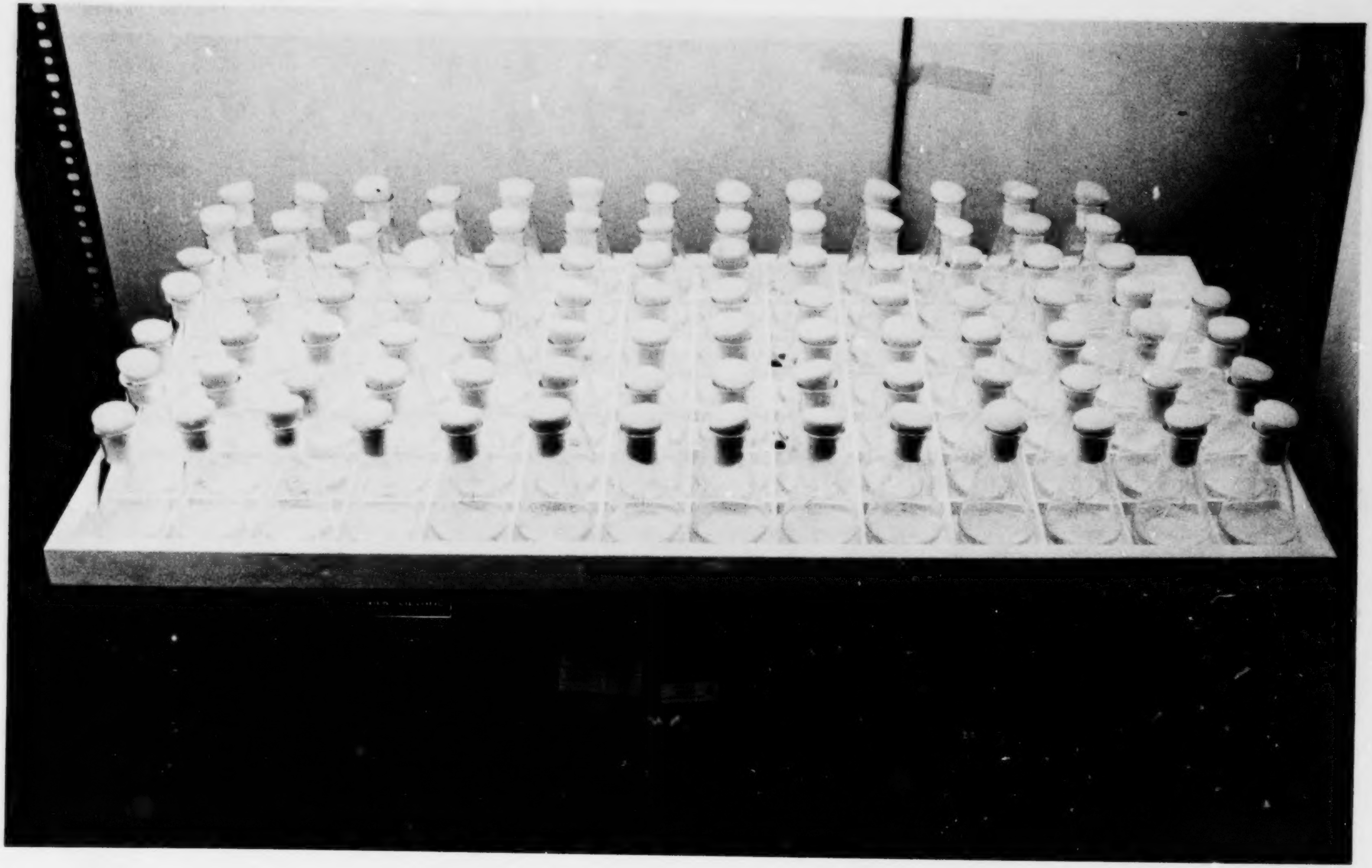

Figure 1a. Shaker platform with 125 ml Erlenmeyer flasks.

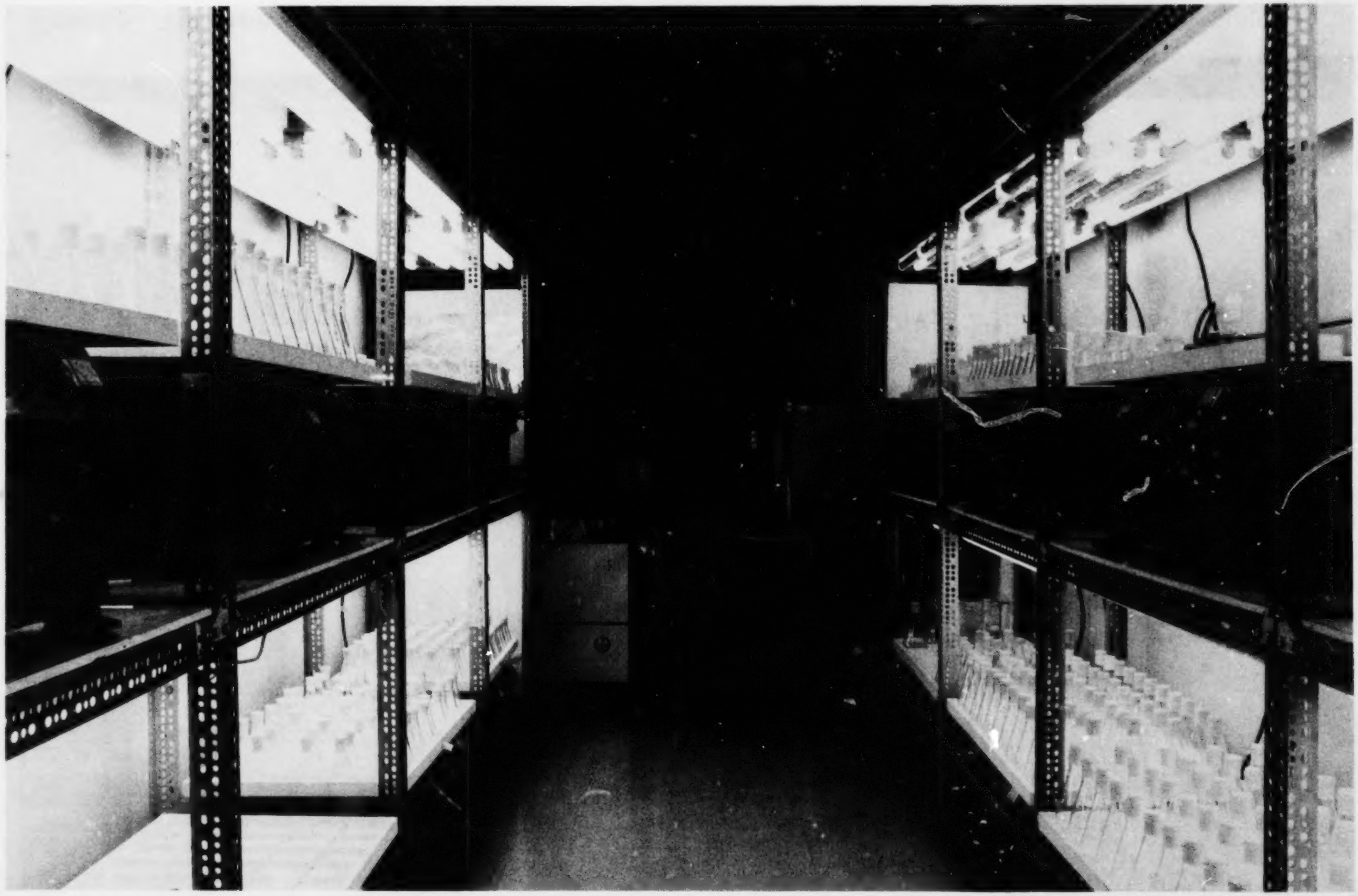

Figure 2. Constant Temperature room.

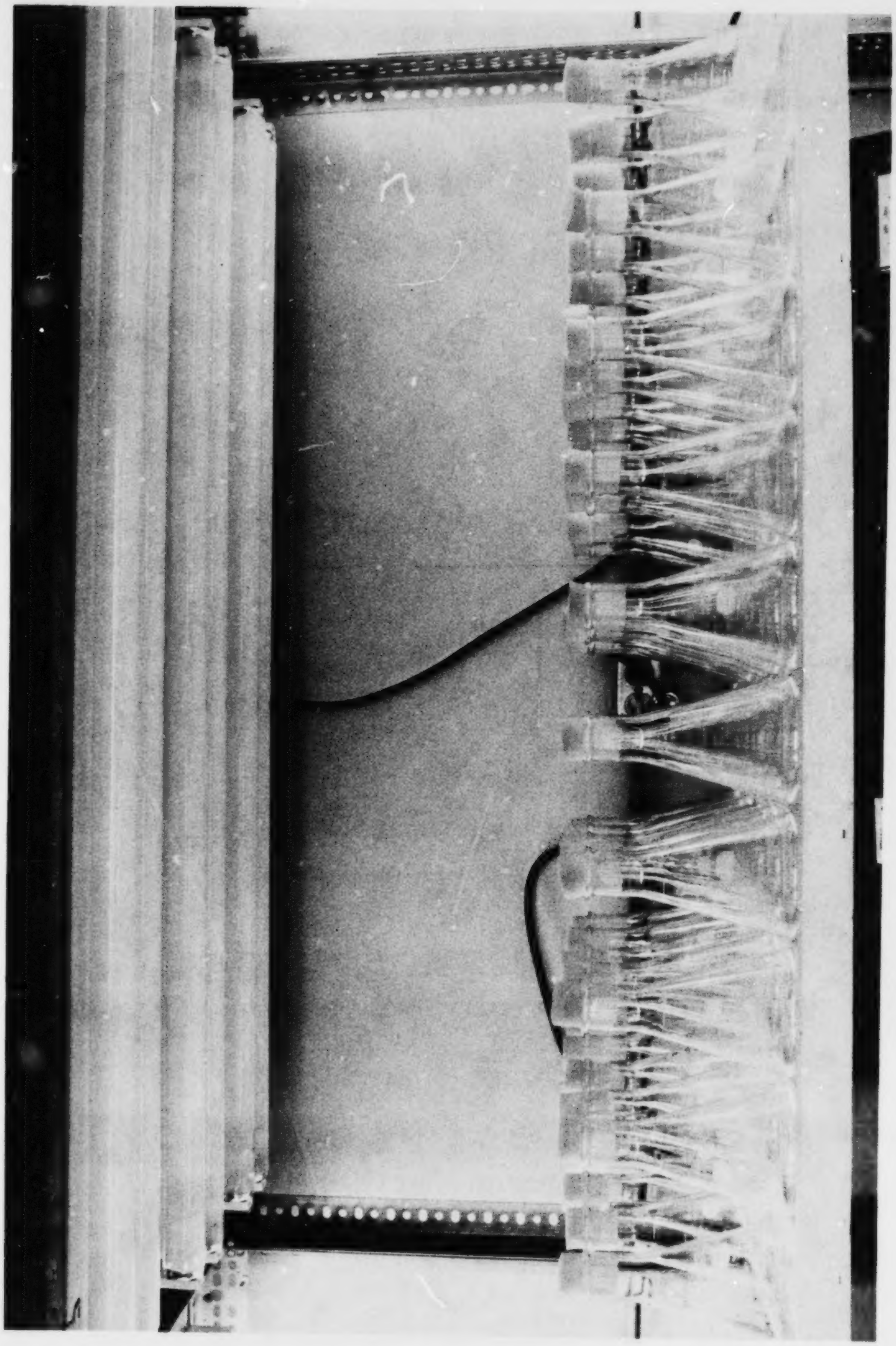

Figure 3. Lightbank and support frame.

4.35 Hemacytometer counting chamber and occular micrometer (used to measure diameter of MCV reference standard).

The above equipment is listed in the order of use preference for monitoring biomass change.

4.36 Oven--Dry heat capable of temperature of 120°C.

4.37 Centrifuge--Capable of relative centrifugal force of at least 1,000 x g.

5.0 SYNTHETIC ALGAL NUTRIENT MEDIUM

Culture medium is prepared as follows: add one ml of each stock solution in 5.1 through 5.7 in the order given to approximately 900 ml of distilled or de-ionized water and then dilute to one liter. Adjust final medium pH to 7.5 ± 0.1 with 0.1 normal sodium hydroxide or hydrochloric acid as appropriate. Immediately filter the pH adjusted medium through a 0.45 μm membrane at a vacuum not to exceed 380 mm (15 inches) mercury or at a pressure not to exceed 1/2 atmosphere (8 psi).

5.1 Sodium Nitrate Stock Solution: Dissolve 12.750 g $NaNO_3$ in 500 ml distilled water.

5.2 Magnesium Chloride Stock Solution: Dissolve 6.082 g $MgCl_2 \cdot 6H_2O$ in 500 ml distilled water.

5.3 Calcium Chloride Stock Solution: Dissolve 2.205 g $CaCl_2 \cdot 2H_2O$ in 500 ml distilled water.

5.4 Micronutrient Stock Solution: Dissolve in 500 ml distilled water:

92.760 mg H_3BO_3
207.690 mg $MnCl_2 \cdot 4H_2O$
1.635 mg $ZnCl_2$
79.880 mg $FeCl_3 \cdot 6H_2O$

0.714 mg $CoCl_2 \cdot 6H_2O$
3.630 mg $Na_2MoO_4 \cdot 2H_2O$
0.006 mg $CuCl_2 \cdot 2H_2O$

150.000 mg $Na_2EDTA \cdot 2H_2O$ [Disodium (Ethylenedinitrilo) tetraacetate]

5.5 Magnesium Sulfate Stock Solution: Dissolve 7.350 g $MgSO_4 \cdot 7H_2O$ in 500 ml distilled water.

5.6 Potassium Phosphate Stock Solution: Dissolve 0.522 g K_2HPO_4 in 500 ml distilled water.

5.7 Sodium Bicarbonate Stock Solution: Dissolve 7.500 g $NaHCO_3$ in 500 ml distilled water.

If desired, reagent salts 5.1 through 5.4 can conveniently be combined into one 500 ml stock solution.

5.71 Final concentration of macronutrients as salts and elemental concentration (mg l^{-1}) of distilled or de-ionized water.

compound	concentration (mg l^{-1})	element	concentration (mg l^{-1})
$NaNO_3$	25.500	N	4.200
$MgCl_2 \cdot 6H_2O$	12.164	Mg	2.904
$CaCl_2 \cdot 2H_2O$	4.410	Ca	1.202
$MgSO_4 \cdot 7H_2O$	14.700	S	1.911
K_2HPO_4	1.044	P	0.186
$NaHCO_3$	15.000	Na	11.001
		K	0.469
		C	2.143

5.72 Final concentration of micronutrients as salts and elemental concentration (μg l^{-1}) in distilled or de-ionized water.

compound	concentration (μg l^{-1})	element	concentration (μg l^{-1})
H_3BO_3	185.520	B	32.460
$MnCl_2 \cdot 4H_2O$	415.610	Mn	115.374
$ZnCl_2$	3.271	Zn	1.570
$CoCl_2 \cdot 6H_2O$	1.428	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.260	Mo	2.878
$FeCl_3 \cdot 6H_2O$	160.000	Fe	33.051
$Na_2EDTA \cdot 2H_2O$	300.000	--	------

5.73 Storage of culture medium--Culture medium must be filter-sterilized (Sec. 5.0) or autoclaved. It is also recommended that uninoculated sterile medium be stored in the dark at 4°C to avoid any (unknown) photochemical changes.

6.0 TEST ALGA

The recommended test alga Selenastrum capricornutum Printz is a green alga (chlorophyceae) of the order chlorococcales. This alga was isolated from the River Nitelva, in the County of Akershus, Norway, by Olav M. Skulberg, Norwegian Institute for Water Research, 1959. Many green algae such as Chlorella, Scenedesmus, and Ankistrodesmus occur in waters of the most diversified composition. Selenastrum belongs to this group of ubiquitous algae which have a wide tolerance towards environmental conditions (Rodhe, 1978). Selenastrum capricornutum is characterized by its unicellular habit in which the cells are in a non-motile condition throughout their entire life cycle. These attributes allow this alga to be enumerated by an electronic particle counter.

6.1 Source of test alga--Available from the Environmental Protection Agency, Corvallis Environmental Research Laboratory, Special Studies Branch, 200 SW 35th Street, Corvallis, Oregon 97330.

This test alga is also available (ATCC 22662) from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852.

6.2 Maintenance of stock culture:

6.21 Medium--See section 5.0

6.22 Incubation conditions--24 ± 2°C.

Under continuous "Cool-White" fluorescent lighting at 4304 lumens (400 ± 10% ft-c), shaken continuously at 100 rpm.

6.3 Culture transfer--Upon receipt of the algal culture, a portion should be aseptically transferred to the algal culture medium as prepared in section 5.0. The volume transferred is not critical (approximately 1.0 ml), however, be sure enough cells are included to overcome significant growth lag. (i.e., 1.0 ml of algal culture in 25 ml of medium in a 125 ml Erlenmeyer flask if not continuously shaken or 1.0 ml culture added to 50 ml in 125 ml flask when shaken continuously.) The rest of the culture can be maintained up to six months in a dark refrigerator at 4°C.

6.4 Subsequent stock transfers--Weekly aseptic routine stock transfer is recommended to maintain a continuous supply of "healthy" cells for experimental work. Extreme care should be exercised to avoid contamination of stock cultures. To retain a unialgal culture over a long period of time it is advantageous to prepare a semi-solid medium containing 1.0% agar. This semi-solid medium is placed in sterile Petri plates. A portion of a liquid algal culture is streaked onto it and incubated under conditions in 6.22. Algae should be transferred onto fresh plates every four weeks. Fresh liquid cultures should be started by transfer of a single algal colony to liquid medium at four week intervals. For regular inoculation, liquid cultures are superior since agar cultures usually are not uniform because the cell layers on the agar surface are differentially supplied with light and nutrients (as a result of shading and diffusion).

6.5 Preparation of inoculum--Rinse algal inoculum free of culture medium as follows: Fill centrifuge tube with 7-10 day stock culture and centrifuge at 1000 x g for 5 minutes. Decant the supernatant and resuspend the cells in sterilized distilled water. Repeat the centrifugation and decantation step and resuspend the cells in distilled water prior to determining the initial cell concentration.

After determining the initial algal cell counts the following equation can be used to prepare the inoculum:

$$\frac{\begin{array}{c}\text{(A)}\\ \text{FINAL VOLUME}\\ \text{OF INOCULUM}\\ \text{(ml)}\end{array} \times \begin{array}{c}\text{(B)}\\ \text{FINAL CONCENTRATION}\\ \text{OF INOCULUM}\\ \text{IN TEST FLASK}\\ (\text{cells ml}^{-1})\end{array} \times \begin{array}{c}\text{(C)}\\ \text{VOLUME OF SOLUTION}\\ \text{IN TEST FLASKS}\\ \text{(ml)}\end{array}}{\begin{array}{c}\text{(D)}\\ \text{INITIAL ALGAL CELL COUNTS } (\text{cells ml}^{-1})\\ \text{IN THE WASHED STOCK CULTURE}\end{array}} = Q$$

Example: 180 flasks containing 100 ml of solution (C) are required for the test. Each flask is to be inoculated with 1000 cells ml^{-1} (B) final concentration. 200 ml of suspended algal cells (A) should be prepared to insure an adequate amount of inoculum. The product of A, B, and C is divided by the initial algal cell count (D). The resulting quotient (Q) indicates the volume (ml) of the initial stock culture suspension (D) to be added to the volumetric flask (A) before bringing the solution up to volume. This inoculum solution should contain a final concentration of 100,000 ± 10% cells ml^{-1}, one ml of which (when added to 100 ml of test solution) results in a final algal cell concentration in the test flask of 1000 cells ml^{-1}.

7.0 TEST CONDITIONS

7.1 Temperature--24 ± 2°C.

7.2 Illumination--Continuous "Cool-White" fluorescent lighting 4304 lumens (400 ± 10% ft-c).

7.3 Gas exchange--Free exchange through foam plugs, shaken at least once daily (see sample to volume reference in section 4.21) or at the preferred rate of 100 rpm.

8.0 PROCEDURE

8.1 Preparation of glassware--The recommended procedure is as follows: All cylinders, flasks, bottles, centrifuge tubes and vials are washed with detergent and rinsed thoroughly with tap water. This is followed by a rinse with 10% solution (by volume) of reagent hydrochloric acid (HCl); vials and centrifuge tubes are filled with the 10% HCl solution and allowed to remain a few minutes; all larger containers are filled to about one-tenth capacity with HCl solution and swirled so that the entire inner surface is bathed. After the HCl rinse, the glassware is neutralized with a saturated solution of Na_2CO_3, then rinsed five times with tap water followed by five rinses with deionized or distilled water.

Disposable pipettes may be used to eliminate the need for pipette washing and to minimize the possibility of contamination.

Cleaned glassware is dried at 50°C in an oven and is then stored either in closed cabinets or on open shelves with the tops covered with aluminum foil.

The recommended procedure for culture flask preparation is as follows: Brush the inside of flasks with a stiff bristle brush to loosen any attached materials. Wash with non-phosphate detergent and rinse thoroughly with tap water. Rinse with a 10% solution (by volume) of reagent grade hydrochloric acid (HCl) by swirling the HCl solution so that the entire surface is covered. Neutralize with saturated sodium carbonate solution (Na_2CO_3). The glassware should be rinsed thoroughly with distilled water. If an electronic particle counter is to be used, the final rinse must be with 0.22 micrometer membrane filtered distilled water. Dry the flasks in an oven at 50°C. Insert foam plugs and autoclave for 20 minutes at 1.1 kg cm^2 and 121°C. The cooled flasks can be stored in closed cabinets until needed.

8.2 pH Control--To insure the availability of carbon dioxide the pH should be maintained below 8.5. This can be accomplished by (1) using optimum sample to volume ratios; (2) continuously shaking the flask (approximately 100 revolutions per minute); (3) ventilation with air or air/carbon dioxide mixture; and, in extreme cases, by (4) bubbling an air/carbon dioxide mixture through the culture. The growth response of S. capricornutum cultured in algal culture medium adjusted either with sodium hydroxide or hydrochloric acid to obtain initial pH values ranging from 3.0 to 11.0 in single unit increments, is shown in Table 2.

TABLE 2
THE EFFECT OF INITIAL pH UPON THE GROWTH RESPONSE OF S. capricornutum CULTURED IN ASSAY MEDIUM

Initial pH	Maximum yield mg dry wt l^{-1}
3.0	0.20
4.0	0.33
5.0	79.69
6.0	89.30
7.0	87.95
8.0	90.02
9.0	82.32
10.0	101.22
11.0	75.10

The resultant growth suggests that initial pH values ranging between 6.0 and 10.0 have no adverse effect upon the 14-day maximum yield of the test alga, when cultured under free gas exchange conditions.

8.3 Growth parameter--The parameter used to describe growth of the test alga is maximum standing crop expressed as dry weight. The maximum standing crop in any flask is defined as the maximum biomass achieved during incubation. For practical purposes, it may be assumed that the maximum standing crop is obtained within 14 days or whenever the increase in biomass is less than 5% per day.

Growth rate should not be used as a growth parameter in batch cultures since growth rate is indirectly related to external nutrient concentrations. This explains why phytoplankton in natural waters may grow at their maximal rate even when there is not a significant amount of the limiting nutrient in the water. For the same reason, phytoplankton may also grow at different rates even when exposed to the same external nutrient concentration. Therefore, the conventional Monod equation, which predicts growth rate in terms of external nutrient levels, does not adequately describe the growth of phytoplankton. Specific details and the scientific rationale concerning growth rate interactions can be found in the following references: Thomas and Dodson, 1968; Golterman *et al*., 1969; Eppley and Thomas, 1969; Rhee, 1972; Swift and Taylor, 1974.

8.4 Laboratory measurement--After the maximum standing crop has been achieved, the dry weight of algal biomass may be calculated indirectly or determined gravimetrically. If biomass is determined indirectly, the results should be converted to an equivalent dry weight using appropriate conversion factors. For example: Electronic particle counts and associated mean cell volumes (MCV) of *S. capricornutum* can be converted to *calculated dry weight* in mg l^{-1} by the following equation:

$$\underset{(\text{Cells ml}^{-1})}{\text{CELL COUNTS}} \times \underset{\text{micrometers)}}{\overset{\text{MCV}}{\text{(Cubic}}} \times [3.6 \times 10^{-7}] = \underset{\textit{S. capricornutum}\ \text{l}^{-1}}{\text{mg dry weight}}$$

Caution: This equation is valid only when the MCV computer has been calibrated with an appropriate reference particle, i.e. # 13020 60 μm^3 standard verified and supplied by Coulter Electronics Inc., Hialeah, Florida. A *maximum of 199* μm^3 can be read directly from the MCV computer. The MCV of *S. capricornutum* can increase beyond 199 μm^3 when cultured in test waters containing heavy metals, pesticides and complex industrial wastes. Adjustment of either the amplification or aperture current will electronically reduce or increase the mean cell volume readout by a constant factor. This allows calibration or scale readouts for particles greater than 199 μm^3. A change in amplification setting from ½ to 1.0 results in a multiplication factor of 2.0 (1.0 ÷ ½), i.e., a direct scale readout of 110 μm^3 at an amplification setting of 1.0 is actually 220 μm^3 (110 × 2).

The MCV calibration for Coulter Counter models ZB, ZBI and ZF is presented in appendix 11.5.

8.5 Biomass monitoring--Several methods may be used, but they must always be related to dry weight. The following methods are listed in order of preference.

8.51 Dry weight--Indirect electronic particle counting

The principle of operation is as follows: the S. capricornutum cells are suspended in a 1% sodium chloride electrolyte in a ratio of 1.0 ml cell suspension to 9 ml of 0.22 µm filtered saline (10:1 dilution). The resulting suspension is passed through a 100 µm diameter aperture. Each cell that passes through the aperture causes a voltage drop proportional to its displaced electrolyte volume which is recorded as a count. The knowledge of both the number of particles (cells) per unit volume of sample (usually 1/2 ml) and the change in mean particle (cell) volume, allow changes in cell biomass (mg dry wt l^{-1}) to be calculated reproducibly and accurately, using the equation as outlined in section 8.4.

8.52 Dry weight--Gravimetric

Method I--A suitable portion of algal suspension is centrifuged, the sedimented cells washed three times in distilled water, transferred to tared crucibles or aluminum cups, dried overnight in a hot air oven at 70-75°C and weighed. This method is more sensitive than Method II, but is open to error through loss of cells during washing.

Method II--This method involves filtering a measured portion of algal suspension through a tared Millipore® filter. The filter recommended is type BD with an 0.60 micrometer pore size.

The method is as follows:

(1) Dry filters for two hours at 70°C in an oven. (Temperatures above 75°C will close the membrane pores).

(2) Cool filters in a desiccator containing desiccant for at least one hour before weighing.

(3) Filter a suitable measured aliquot of the culture under a vacuum of 380 mm of mercury (or at a pressure not to exceed 1/2 atmosphere). Normally 10 ml is sufficient, but in thin cultures more may be required.

(4) Rinse the filter funnel with 50 ml distilled water using a wash bottle and allow the rinsings to pass through the filter. This serves to transfer all of the algae to the filter and washes the nutrient salts through the filter.

(5) Dry the filter to constant weight at 70°C, cool in a desiccator for one hour and weigh.

8.53 Chlorophyll *a*--*In vivo* fluorescence of algal chlorophyll has been used with many types of algae and has proved particularly useful with *S. capricornutum* and with indigenous algae or filamentous forms not easily measured at low concentrations by direct microscopic, gravimetric dry weight and absorbance methods. This method is sensitive and can be quickly performed. However, chlorophyll to cell mass ratio may vary significantly with growth in natural waters having different chemical composition (Kuhl and Lorenzen, 1964). Chlorophyll measurement is unsatisfactory to assess the toxic or stimulatory effects of complex wastes which may absorb and fluoresce in the same spectral region. In vivo fluorescence measurements can aid in evaluating increases in cell biomass attributed to increased growth in specific test waters, but should not be used to predict universal chlorophyll a to dry weight biomass relationships.

8.54 Direct microscopic enumeration--Hemacytometer

8.55 Absorbance--The use of turbidity for algal cell measurements is strongly discouraged. Table 3 presents the relationship between the different biomass monitoring methods. Note that there was no definition between 5,000 and 115,000 cells ml^{-1} when assessed as absorbance utilizing a spectrophotometer at 750 nm (cell path of 1 cm).

9.0 DATA ANALYSIS

9.1 Introduction--The fundamental measure used in this Algal Assay: Bottle Test to describe algal growth is the maximum dry weight mg l^{-1} (standing crop) produced during the 14-day incubation period. Other biomass indicators such as those listed in section 8.5 may be used; however, all results presented must include experimentally determined conversion factors between the indicator used and the dry weight of S. capricornutum obtained.

9.2 Confidence intervals--The maximum standing crop should be presented with the confidence interval indicated. The calculation of confidence interval for the average values presented must be based on at least three samples. Consequently, a minimum of three replications per sample and/or sample treatment must be analyzed when a source water is studied. The results of these three replicates are then used to calculate the standard deviation. Confidence intervals are based upon the standard deviation (σ).

TABLE 3
RESULTS OF BIOMASS ASSESSMENT TECHNIQUES PRODUCED IN ALGAL ASSAY PROCEDURE LABORATORY CLASSES

CELL COUNTS (Cells ml^{-1}) ELECTRONIC			DRY[d] WEIGHT mg 1^{-1}	ABSORBANCE[e]	FLUORESENCE		
ORIGINAL[a]	STUDENT[b]	HEMACYTOMETER[c]		1 cm cell path @ 750 Nm	TURNER[f] Mod 111	TURNER[g] DESIGN	PRODUCTIVITY[h] CLASSIFICATION
1,949,125	2,290,490	2,475,000	28.40	0.060	11,400	570	
1,320,000	1,239,578	2,125,000	14.50	0.056	11,500	590	
1,320,000	1,698,888	1,075,000	21.51	0.060	10,750	632	
1,023,815	1,159,050	950,000	15.00	0.043	11,250	340	HIGH PRODUCTIVITY
1,023,815	887,373	1,135,000	10.80	0.045	10,744	280	
501,737	573,902	680,000	7.50	0.021	2,400	180	(6.10-20.00 mg dry weight 1^{-1})
501,737	513,434	360,000	6.50	0.024	1,829	180	
458,000	533,500	470,000	6.60	0.015	3,650	234	
458,000	521,256	675,000	6.46	0.019	3,150	240	
115,226	108,580	85,000	1.30	0.005	20	34	MODERATELY HIGH PRODUCTIVITY
115,226	117,331	110,000	1.50	0.005	53	26	(0.81-6.00 mg dry weight 1^{-1})
57,000	58,647	85,000	0.68	0.005	330	27	MODERATE PRODUCTIVITY
50,031	48,978	305,000	0.60	0.005	10	12	(0.11-0.80 mg dry weight 1^{-1})
50,031	52,010	65,000	0.70	0.005	14	12	
10,340	9,070	10,000	0.10	0.000	18	0.5	
10,473	10,868	63,000	0.10	0.000	3	2.6	LOW PRODUCTIVITY
10,473	13,208	10,000	0.20	0.000	3	1.5	
5,846	5,791	4,500	0.07	0.000	24	2.2	(0.00-0.10 mg dry weight 1^{-1})
5,846	5,543	5,000	0.07	0.000	26	2.3	

[a] Counts of Selenastrum capricornutum produced on a Coulter Electronic Particle Couter Model ZBI. Cell suspensions were prepared by EPA ALGAL STAFF. [b] Electronic cell counts taken by students. [c] Hemacytometer counts taken by students. [d] Calculated dry weights based on student-derived cell counts X mean cell volume X 2.0 X 10^{-7}. [e] Optical density. f and g relative fluorescence units of chlorophyll a non-extracted Selenastrum cells. [h] Productivity classifications are from Miller, Maloney, and Greene, (1974).

σ = confidence interval of 66.6 percent

2σ = confidence interval of 95.0 percent

3σ = confidence interval of 99.0 percent

$$\sigma = \pm \sqrt{\frac{\Sigma x^2 - (\Sigma x)^2/n}{n - 1}}$$

Example of calculation--Taking data (dry weight) from Table 4 for the triplicate set of control flasks for day fourteen, the constants in the equation are as follows:

$x_1 = 0.14 \qquad x_1{}^2 = 0.0196$

$x_2 = 0.14 \qquad x_2{}^2 = 0.0196$

$x_3 = 0.13 \qquad x_3{}^2 = 0.0169$

$n = 3$

Therefore: $\Sigma x^2 = 0.0561$

$(\Sigma x)^2 = (0.41)^2 = 0.1681$

$$\frac{(\Sigma x)^2}{n} = \frac{0.1681}{3} = 0.0560$$

$$\sigma = \pm \sqrt{\frac{(0.0561) - (0.0560)}{2}} = \sqrt{0.0005} = \pm 0.007$$

$2\sigma = \pm 0.014$

$3\sigma = \pm 0.021$

TABLE 4
TYPICAL REPORT OF ASSAY RESULTS

	calculated dry weight mg l^{-1}											
	control				control + 0.05 mg P l^{-1}				control + 1.0 mg N l^{-1}			
days	1	2	3	Avg	1	2	3	Avg	1	2	3	Avg
0	.02	.02	.02	.02	0.02	0.02	0.02	0.02	.02	.02	.02	.02
3	.09	.07	.08	.08	1.62	1.61	1.56	1.60	.10	.08	.10	.09
5	.13	.13	.11	.12	6.10	6.50	6.61	6.40	.12	.14	.13	.13
7	.13	.12	.11	.12	7.60	7.75	7.65	7.67	.14	.16	.16	.15
10	.14	.13	.12	.13	8.75	8.80	8.70	8.75	.14	.15	.15	.15
14	.14	.14	.13	.14	8.80	8.85	8.75	8.80	.13	.14	.16	.14

The following is an example of how one determines the required number of replicates: Considering the design of an experiment to compare two media, one of known strength (m_1) which will produce a maximum standing crop of about 8.75 mg dry weight l^{-1} and another medium (m_2) expected to produce a greater standing crop. The "null hypothesis," which one expects to disprove, is that $m_2 \leqq m_1$, i.e., that the unknown medium produced a standing crop not larger than the known medium. The "alternative hypothesis," which one expects to prove, is that $m_2 > m_1$, i.e., that the unknown medium produces a greater standing crop than the known medium.

How many replicate flasks should be used? The answer can be found by first answering the following five questions and then consulting Table 5.

Question 1: "What significance level, α, should be used?" For this example we shall use the significance level $\alpha = 0.05$, i.e., if the two media are the same strength ($m_1 = m_2$) there will be one chance in twenty that the experiment will result in the erroneous conclusion that the known medium is weaker ($m_1 < m_2$).

Question 2: "What is the smallest difference, $\delta = m_2 - m_1$ which must be detected?" The known medium will produce a standing crop of about $m_1 = 8.75$ mg dry wt. l^{-1}. Suppose the other medium must produce a 10% greater crop (9.62 mg dry wt. l^{-1}) to be "significantly" stronger, i.e., the smallest difference which must be detected is about $\delta = m_2 - m_1 = 9.62 - 8.75 = 0.87$ mg dry wt l^{-1}.

Question 3: "With what probability must a difference of δ_o (= 0.87 mg dry wt l^{-1}) be detected by the experiment?" Suppose a probability of detection of 0.90 is desired, i.e., if the true difference in the standing crops of the media is 0.87 mg dry wt l^{-1}. There is a 90 percent chance the experiment will detect the difference (lead to a conclusion that the known medium is weaker). Conversely, there is a 10% chance that the experiment will fail to detect a difference of 0.87 mg dry wt l^{-1}. Denote the probability of detection as $1 - \beta = 0.90$.

Question 4: "What is the standard deviation, s, of an individual observation?" (Note that this is not the same as the standard error of a mean of several observations.) There would probably be some information about the standard deviation from a prior experiment with the "known" medium. For this example assume that previous experience indicates a standard deviation approximately 0.40 mg dry wt l^{-1}.

Question 5: "Does the alternative hypothesis specify a 'one-tail' alternative ($\delta > 0$, $m_2 > m_1$) or a 'two-tail' alternative ($\delta \neq 0$, $m_2 \neq m_1$)?" In this example it is assumed the findings will be significant only if the unknown medium produces a greater standing crop than the known medium; thus the alternative hypothesis specifies a one-tail alternative $\delta > 0$, $m_2 > m_1$.

TABLE 5

AID IN COMPUTING SAMPLE SIZES REQUIRED TO DETECT PRESCRIBED DIFFERENCES BETWEEN AVERAGES

Notation:

α	Significance level of the test
δ_o	Smallest detectable or significant difference
$1-\beta$	Probability of declaring $\delta \neq 0$ if $\delta = \delta_o$
s	Sample estimate of the standard deviation of an observation
d	$= 0.7071\ \delta_o / s$

ONE TAIL TEST TABLES:

For $\alpha = .01$ add 2 to the tabled value to get the number of replicates; for $\alpha = .05$ add 1 to the tabled value to get the number of replicates.

$\alpha = .01$

d \ 1-β	.50	.60	.70	.80	.90	.95	.99
.1	542	666	813	1004	1302	1578	2165
.2	136	167	204	251	326	395	542
.4	34	42	51	63	82	99	136
.6	16	19	23	28	37	44	61
.8	9	11	13	16	21	25	34
1.0	6	7	9	11	14	16	22
1.2	4	4	6	7	10	11	16
1.4	3	4	5	6	7	9	12
1.6	3	3	4	4	6	7	9
1.8	2	3	3	4	5	5	7
2.0	2	2	3	3	4	4	6
3.0	1	1	1	2	2	2	3

$\alpha = .05$

d \ 1-β	.50	.60	.70	.80	.90	.95	.99
.1	271	361	471	619	857	1083	1578
.2	68	91	118	155	215	271	395
.4	17	23	30	39	54	68	99
.6	8	11	14	18	24	31	44
.8	5	6	8	10	14	17	25
1.0	3	4	5	7	9	11	16
1.2	2	3	4	5	6	8	11
1.4	2	2	3	4	5	6	9
1.6	2	2	2	3	4	5	7
1.8	1	2	2	2	3	4	5
2.0	1	1	2	2	3	3	4
3.0	1	1	1	1	1	2	2

TABLE 5 (continued)

TWO TAIL TEST TABLES:

For $\alpha = .01$ add 2 to the tabled value to get the number of replicates; for $\alpha = 0.05$ add 1 to the tabled value to get the number of replicates.

$\alpha = .01$

1-β d	.50	.60	.70	.80	.90	.95	.99
.1	664	801	962	1168	1488	1782	2404
.2	166	201	241	292	372	446	601
.4	42	51	61	73	93	112	151
.6	19	23	27	33	42	50	67
.8	11	13	16	19	24	28	38
1.0	7	9	10	12	15	18	25
1.2	5	6	7	9	11	13	17
1.4	4	5	5	6	8	10	13
1.6	3	4	4	5	6	7	10
1.8	3	3	3	4	5	6	8
2.0	2	3	3	3	4	5	7
3.0	1	1	2	2	2	2	3

If we must estimate σ from our sample and use Student's t, then we should add 4 to the tabulated values to obtain the approximate required sample size. (If we are comparing two product averages, add 2 to the tabulated values, to obtain the required size of each sample. For this case, we must have $\sigma_A = \sigma_B$.)

$\alpha = .05$

1-β d	.50	.60	.70	.80	.90	.95	.99
.1	385	490	618	785	1051	1300	1838
.2	97	123	155	197	283	325	460
.4	25	31	39	50	66	82	115
.6	11	14	18	22	30	37	52
.8	7	8	10	13	17	21	29
1.0	4	5	7	8	11	13	19
1.2	3	4	5	6	8	10	13
1.4	2	3	4	5	6	7	10
1.6	2	2	3	4	5	6	8
1.8	2	2	2	3	4	5	6
2.0	1	2	2	2	3	4	5
3.0	1	1	1	1	2	2	3

$u + (z_{1-\alpha}{}^{+}{}_{1-\beta})^{2}/d^{2}$, where z_x denotes the cumulative distribution function of the standard normal (0,1) distribution.

Source: Experimental Statistics, by Mary G. Natrella, National Bureau of Standards Handbook 91, U. S. Government Printing Office, Washington, DC. The tables above are Tables A-9 and A-8, respectively, from this reference.

Therefore, a one-tail test (Table 5) would be used. (A two-tail alternative would require a two-tail test.)

In summary, the answers to the questions above have provided the following values:

(1) $\alpha = 0.05$ = significance level

(2) $\delta_o = 0.87$ mg dry wt. 1^{-1} = smallest "significant" difference

(3) $1 - \beta = 0.90$ = probability of detecting smallest significant difference.

(4) $s = 0.40$ mg dry wt 1^{-1} = standard deviation

(5) Alternative hypothesis specifies a one-tail test.

We can now compute the value of "d" and find the required number of replicates from Table 5:

$$\begin{aligned} d &= 0.7071\ \delta_o/s \\ &= (0.7071) \times (0.87)/(0.40) \\ &= 1.54 \end{aligned}$$

Entering the One-Tail test tables with these values we find the number of replicates should be between 5 + 1 (corresponding to $d = 1.4$) and 4 + 1 (corresponding to $d = 1.6$)*. One should use quadratic interpolation in the table, but linear interpolation produces an approximate result: 6 replicates. Note that only 4 replicates would have the desired probability of detecting the difference if $d = 2.0$, i.e., if $\delta_o = \frac{ds}{0.7071} = \frac{(2.00)(0.40)}{0.7071} = 1.13$. That is, 4 replicates would have a 90% chance of detecting a difference of 1.13 mg dry wt. 1^{-1}, a 13% increase in standing crop, whereas 6 replicates are required to ensure a 90% chance of detecting a 10% increase in standing crop. These figures assume the validity of the estimate of the standard deviation.

---

* Note that the tabled value is not the number of replicates; one must add 1 to the tabled values in the $\alpha = 0.05$ table and 2 to the tabled values in the $\alpha = 0.01$ table.

9.3 Rejection of outliers--An "outlier" among replicate observations is one whose deviation from the mean is far greater than the rest in absolute value and perhaps lies three or four standard deviations or further from the mean. The outlier is a peculiarity and indicates a data point which is not at all typical. It follows that an outlier should be submitted for particularly careful examination to see if the reason for its peculiarity can be determined.

Rules have been proposed for rejecting outliers, i.e., for deciding to remove the observation(s) from the data, after which the data are re-analyzed with these observations. Automatic rejection of outliers is not always a wise procedure. Sometimes an outlier is providing information which other data points cannot since it arises from an unusual combination of circumstances which may be of vital interest and requires further investigation rather than rejection. As a general rule, outliers should not be rejected out-of-hand unless they can be traced to specific causes, e.g., errors in recording observations or in setting up apparatus. Otherwise, careful investigation is in order. (The above was adapted from section 3.8 of Applied Regression Analysis by N. R. Draper and H. Smith, John Wiley and Sons, 1968.)

The following test may be applied for rejecting outliers:

1. Rank order the data in the group containing the outliers (all observations in the group are supposedly treated alike):

$$x_1 \leqq x_2 \leqq . . . x_n$$

2. Compute the appropriate criterion:

If x_1 is the outlier $c = \frac{x_2 - x_1}{x_n - x_1}$

If x_n is the outlier $c = \frac{x_n - (x_n - 1)}{x_n - x_1}$

3. If c exceeds the critical value opposite "n" in Table 6, reject the outlier.

TABLE 6
CRITICAL VALUES FOR DETERMINING OUTLIERS

n	Critical values	
	$\alpha = 0.05$	$\alpha = 0.01$
3	0.941	0.988
4	0.765	0.889
5	0.642	0.780
6	0.560	0.698
7	0.507	0.637

Example--Suppose the following replicate dry wt mg l^{-1} observations were made: 9.8, 4.7, 8.4, 8.0, 8.4, and 7.9. The value 4.7 is suspected to be an outlier. Rank order the data.

x_1	x_2	x_3	x_4	x_5	x_6	$n = 6$
4.7	7.9	8.0	8.4	8.4	9.8	

The criterion is as follows:

$$c = \frac{x_2 - x_1}{x_n - x_1} = \frac{7.9 - 4.7}{9.8 - 4.7} = \frac{3.2}{5.1} = 0.63$$

Since n = 6, this value is significant at the $\alpha = 0.05$ level ($0.63 > 0.560$), but not at the $\alpha = 0.01$ level ($0.63 < 0.698$).

The experimenter who is willing to discard 5% of all his good data would discard the observation 4.7 as an outlier. The experimenter who is willing to discard only 1% of his good data would keep the observation unless he can determine an experimental reason for rejecting it.

4. If there are two suspected outliers (say x_1 and x_n or x_1 and x_2), the test may be repeated; apply it to the "worst" outlier first.

5. Note that the regular use of this procedure will result in discarding five percent (if $\alpha = 0.05$) or one % (if $\alpha = 0.01$) of all one's good (valid) observations.

10.0 EXPERIMENTAL DESIGN AND ANALYSIS

10.10 Nutrient limitation

10.11 Introduction--The "Algal Assay: Bottle Test" can be used to define nutrient limitation in natural waters, whether this limitation is due to nitrogen, phosphorus or trace element deficiency. This is accomplished by an experimental design which incorporates an internal check and balance system centered around the growth response of *S. capricornutum* to singular and combined additions of nitrogen, phosphorus, and EDTA to the test waters. The growth responses obtained are then evaluated to ascertain the limiting nutrient(s).

10.12 Experimental design--The following series of nutrient and chelator additions in Table 7 are considered as the *minimum* necessary to determine the nutrient status of an *unevaluated* test water. They are: the test water control and final spike concentrations equivalent to mg l^{-1} in each test flask.

TABLE 7
BASIC EXPERIMENTAL DESIGN TO DEFINE NUTRIENT LIMITATION

Control

Control + 0.05 mg P l^{-1} as K_2HPO_4

Control + 1.00 mg N l^{-1} as $NaNO_3$

Control + 0.05 mg P l^{-1} + 1.00 mg N l^{-1}

Control + 1.00 mg Na_2 EDTA l^{-1} as Disodium (Ethylenedinitrilo) tetraacetate

Control + 0.05 mg P l^{-1} + 1.00 mg Na_2 EDTA l^{-1}

Control + 1.00 mg N l^{-1} + 1.00 mg Na_2 EDTA l^{-1}

Control + 0.05 mg P l^{-1} + 1.00 mg N l^{-1} + 1.00 mg Na_2 EDTA l^{-1}

Each nutrient chelator addition was selected based on past experience of evaluation effectiveness. For example: the 0.05 mg P l^{-1} spike was chosen to insure the saturation (excess) of phosphorus within the sample, which is necessary to drive the system to the secondary limiting nutrient. Each μg P l^{-1} will support 0.430 ± 20% mg dry weight l^{-1} of *S. capricornutum* if other constituents are not growth limiting. Therefore, the 0.05 mg P l^{-1} additions should support additional growth in the control test water up to a maximum of 21.50 mg dry wt l^{-1} depending upon the availability of other essential nutrients (primarily nitrogen) within the test water.

Similar rationale pertains to the selected nitrogen addition of 1.0 mg l^{-1} which should support an additional increase in biomass up to 38.00 mg dry wt l^{-1} (0.038 ± 20% mg dry wt per mg N l-1) or to that level which can be supported by (in most cases) the available phosphorus content in the test water.

The combined nitrogen and phosphorus addition will generally support growth relative to the phosphorus content in the water. This reflects the excess nitrogen conditions which are intentionally introduced into the test water, i.e., 38.00 mg dry wt l^{-1} for the nitrogen spike versus 21.50 mg dry wt l^{-1} due to the phosphorus addition.

The Na_2 EDTA chelator addition of 1.00 mg l^{-1} was selected after the evaluation of additions of 0.3, 1.0, 5.0 and 10.0 mg l^{-1} upon the growth response of _S_. _capricornutum_ in both assay medium and selected natural waters. The lowest addition (0.3 mg l^{-1}) was capable of insuring trace element availability in the culture medium (see sec. 5.0) but was not sufficient to complex the heavy metals present in many natural waters. Na_2 EDTA addition in excess of 1.0 mg l^{-1} caused complexion of essential macronutrients (i.e. Ca and Mg) depressing growth relative to the N and P content of the test waters.

10.13 Essential background data--The _minimum_ chemical data necessary to evaluate the assay response to define nutrient limitation are: Initial pH; Total phosphorus; Ortho-P; NO_2; NO_3; NH_3 and total Kjeldahl nitrogen.

10.14 Test conditions--Each test flask is inoculated to contain a final concentration of 1,000 cells ml^{-1} of _S_. _capricornutum_ and is incubated at 24 ± 2°C under 4304 lumens (400 ± 10% ft-c) and shaken once daily or continuously (see sec. 4.21) for a period of at _least_ 14 days.

10.15 Interpretation of results--_All_ nutrient limitation assay results _must_ be reported as the maximum standing crop (MSC) in mg dry wt l^{-1}. Typical 14-day growth responses representative of phosphorus, nitrogen, trace element and nitrogen plus phosphorus growth limitation are presented.

10.16 Phosphorus limitation--The following growth responses (Table 8A) and the corresponding control test water chemical analysis data (Table 8B) are typical of phosphorus-limited waters.

TABLE 8A
GROWTH RESPONSES REPRESENTATIVE OF PHOSPHORUS LIMITATION

Sample Treatment	MSC (mg dry wt l^{-1})
Control	2.16
Control + 0.05 mg P l^{-1}	5.81
Control + 1.00 mg N l^{-1}	2.30
Control + 1.00 mg N and 0.05 mg P l^{-1}	23.69
Control + 1.00 mg Na_2 EDTA l^{-1}	2.10
Control + 1.00 mg Na_2 EDTA + 0.05 mg P l^{-1}	5.66
Control + 1.00 mg Na_2 EDTA + 1.00 mg N l^{-1}	2.30
Control + 1.00 mg Na_2 EDTA + 0.05 P + 1.00 mg N l^{-1}	24.60

TABLE 8B
CHEMICAL ANALYSIS OF THE PHOSPHORUS LIMITED CONTROL TEST WATER AND PREDICTED N AND P YIELDS (mg l^{-1}).

0.021 mg Total P l^{-1}	
0.006 mg Ortho-P l^{-1}	= 0.006 x 430 = 2.58 ± 20%*
0.368 mg Total N l^{-1}	
0.120 mg $NO_3 + NO_2$-N l^{-1}	
0.040 mg NH_3-N l^{-1}	
0.160 mg TSIN^{-1} ($NO_2 + NO_3 + NH_3$)	= 0.160 x 38 = 6.10 ± 20%*
>26:1 N:P ratio (TSIN ÷ Ortho-P)	

* Predicted yields of *S. capricornutum* based on soluble inorganic nitrogen or phosphorus content of the test water if all other essential nutrients are present in excess.

The ratio of the TSIN to Ortho-P yield factors (38 and 430, respectively) indicates an optimum N:P ratio of ≅ 11:1 for the support of *S. capricornutum*. The N:P ratio can be used as a "guide" to nutrient limitation in most natural waters. That is, waters containing N:P ratios greater than 11:1 may be considered phosphorus limited while those containing N:P ratios less than 11:1 can be considered nitrogen limited for algal growth. Placement into a nitrogen or phosphorus limitation category without actual assay analysis is discouraged. Only assay response to the nutrient and/or chelator additions can verify nutrient limitation and the extent of N and P bioavailability in the test water. The test water used in this example has a N:P ratio of > 26:1. This strongly indicates the potential for phosphorus limitation. The actual assay response confirms the N:P ratio prediction of nutrient limitation in this test water.

Differences in maximum standing crop are not considered statistically different at the 95% (2α) confidence level if they fall within the limits established in Table 1. Therefore, only the responses obtained by addition of phosphorus, singly and in combination, with nitrogen and Na_2 EDTA are considered to be statistically significant in this test water. These responses are directly proportional to the increase in phosphorus, and are secondarily limited by the TSIN content of the test water. For example: 0.160 mg N l^{-1} contained in the control test water can support 6.10 ± 20% mg dry wt l^{-1} of S. capricornutum due to its nitrogen availability, even though the addition of 0.05 mg P l^{-1} was enough phosphorus to support 21.50 mg dry wt l^{-1}.

The phosphorus regulated growth response obtained in the control and in the test waters containing additions of nitrogen and Na_2 EDTA, singly and in combination, should be essentially identical (within ± 20%) in the phosphorus limited test waters. Thus, 12 replicate flasks can be used as built-in check and balance criteria to define the validity and accuracy of the assay results. For example: if the yield in any of these replicate flasks exceeded ± 50% it would immediately be suspect as an *outlier* and in most cases would be discarded.

The *biological availability* of nitrogen and phosphorus in the test water can be *calculated* by dividing the MSC by either the TSIN or Ortho-P yield factors. The MSC obtained with 0.05 mg P l^{-1} addition should be used to calculate nitrogen availability. For example: $5.81 \div 38 = 0.152$ mg available nitrogen l^{-1}. This calculated value compares favorably with the chemically analyzed TSIN value of 0.160 mg l^{-1}. Thus, in this test water *all* of the *TSIN* was *available* for growth of the test alga. This conclusion is important because no other growth factor except phosphorus in the presence of adequate nitrogen is regulating growth in this test water.

The biologically available phosphorus content in this test water is derived by dividing the yield obtained with 1.00 mg N l^{-1} addition by the phosphorus yield coefficient. Thus, the *control plus 1.00 mg N* l^{-1} yield of $2.30 \div 430 = 0.005$ mg available P l^{-1}. This *back calculated* value of 0.005 mg l^{-1} is verification of the chemically analyzed value of 0.006 mg Ortho-P l^{-1}. This biologically reactive phosphorus value (0.005 mg l^{-1}) can also be used to calculate the percentage of bioavailable total phosphorus (0.021 mg l^{-1}) which in this test water is 24% ($0.005 \div 0.021$). The bioavailable nitrogen and phosphorus concentrations in this test water correlate with their chemically analyzed concentrations. *Failure* of a test water to *attain this correlation* can be attributed to: presence of bioavailable organic nutrients; effect of other growth-limiting nutrients; the presence of inhibitory constituents in the test water; and/or unreliable chemical analysis for Ortho-P and TSIN.

10.17 Nitrogen limitation--The following assay growth responses (Table 9A) and corresponding control test water chemical analysis data (Table 9B) are typical of nitrogen limitation in natural test waters.

TABLE 9A
GROWTH RESPONSES REPRESENTATIVE OF NITROGEN LIMITATION

Sample Treatment	MSC (mg dry wt l^{-1})
Control	4.06
Control + 0.05 mg P l^{-1}	4.21
Control + 1.00 mg N l^{-1}	12.68
Control + 1.00 mg N + 0.05 mg P l^{-1}	34.52
Control + 1.00 mg Na_2 EDTA l^{-1}	6.30
Control + 1.00 mg Na_2 EDTA + 0.05 mg P l^{-1}	6.49
Control + 1.00 mg Na_2 EDTA + 1.00 mg N l^{-1}	12.80
Control + 1.00 mg Na_2 EDTA + 1.00 mg N + 0.05 mg P l^{-1}	34.68

TABLE 9B
CHEMICAL ANALYSIS OF THE CONTROL TEST WATER AND CORRESPONDING N:P RATIO WITH PREDICTED YIELDS (mg l^{-1}).

0.072 mg Total P l^{-1}	
0.030 mg Ortho-P l^{-1}	= 0.030 x 430 = 12.90 ± 20%
0.160 mg Total N l^{-1}	
0.055 mg NO_3 + NO_2-N l^{-1}	
0.020 mg NH_3-N l^{-1}	
0.075 mg TSIN l^{-1}	= 0.075 x 38 = 2.85 ± 20%
2.5:1 N:P ratio (TSIN ÷ Ortho-P)	

The growth responses obtained in the control and the control plus nitrogen and/or chelator additions identify nitrogen as the primary growth limiting nutrient. These responses can also be used to define: the bioavailable concentrations of nitrogen and phosphorus; chemical analysis reliability; and nitrogen form utilization.

The following basic assay response analyses were used to define the critical nutrient interactions regulating growth in the test water. The 12.68 mg dry wt l^{-1} obtained by the addition of 1.0 mg N l^{-1} confirms the N:P ratio (2.5:1) limiting nutrient status assigned to this test water. This nitrogen stimulated maximum standing crop divided by the phosphorus yield factor (12.68 ÷ 430 = 0.029 mg l^{-1}) indicates the bioavailable phosphorus content of the test water. The resultant bioavailable concentration of 0.029 mg P l^{-1} is essentially identical to the Ortho-P content of the test water (0.030 mg P l^{-1}).

The bioavailable nitrogen content of the test water was determined by dividing the phosphorus stimulated response by the nitrogen yield factor (4.21 ÷ 38 = 0.111 mg N l^{-1}). This bioavailable nitrogen concentration is 1.5 fold greater than the analyzed TSIN content of the test water. The increase in available nitrogen may be attributed to: unreliable chemical analysis; the utilization of other nitrogen forms (such as organic nitrogen) for the support of *S. capricornutum*; or unreliable assay test results.

The built-in check and balance response yield relationships to the recommended nutrient and/or chelator additions, can be used to define the validity of the calculated 1.5 fold increase of bioavailable nitrogen content in the test water. The first check and balance evaluation is to determine whether the assay yields obtained in the control test water are "statistically equal" to those obtained in the control plus phosphorus test water. The rationale being that; growth in nitrogen limited waters should not be responsive to singular addition of phosphorus. Thus similar yields of 4.06 and 4.21 mg dry wt l^{-1} attained in these test waters confirms the reproducibility and precision of the assay test results. Therefore, the "statistically significant" response of 12.68 mg dry wt l^{-1} obtained by nitrogen addition to the test water (proportional to its bioavailable phosphorus content) validates the primary nutrient limitation status of the test water.

The second algal assay response evaluation to be considered is the identification of the secondary growth-regulating nutrient(s) in the test water. This is accomplished by defining the comparability between the yields obtained in the combined nitrogen and phosphorus spike with those attained in the combined nitrogen, phosphorus, Na_2 EDTA spiked test water. The response of the test alga to combined N and P addition should be "statistically equal" (within ± 10%) to the yield obtained with N, P and Na_2 EDTA addition if a trace-element is not growth limiting. The similar yields obtained of 34.52 and 34.68 mg dry wt l^{-1} respectively, strongly indicate that the growth response is regulated solely by the N and P content in the test water. The comparison of these assay yields with those calculated from the TSIN and Ortho-P content of the test water should identify the secondary growth-regulating nutrient. The TSIN and Ortho-P calculated yields for these test waters are:

TSIN yield = the TSIN content of the test water (0.075 mg l^{-1}) plus that added in the spike (1.00 mg N l^{-1}) multiplied by the nitrogen yield factor (38 x 1.075 = 40.85 ± 20% mg dry wt l^{-1}) equals the MSC which can be supported in the test water.

Ortho-P yield = 0.030 mg P l^{-1} in the control plus 0.050 mg P l^{-1} in the spike multiplied by 430 (0.080 x 430 = 34.40 ± 20% mg dry wt l^{-1})

indicates that a MSC of 34.40 ± 20% mg dry wt l^{-1} can be supported by the phosphorus content of the test water. The MSC obtained by assay analysis of 34.52 and 34.68 mg dry wt l^{-1} to combined N and P additions are statistically equal to those calculated for the phosphorus content in the test waters. Therefore, phosphorus is the secondary growth regulating nutrient.

By evaluating these assay responses we have established: (1) nitrogen is primarily regulating growth; (2) the precision and reproducibility of the assay; (3) phosphorus addition in the presence of excess nitrogen supports growth to its maximum potential; (4) the absence of other growth regulating constituents; and (5) an apparent increase in nitrogen availability beyond that attributed to the TSIN content of the test water.

Establishing that the growth response in a test water is not regulated by an unknown trace-element or inhibitor is prerequisite to defining the reliability of the chemical analysis of TSIN in nitrogen limited waters. This is partly due to the ability of the test alga to metabolize the Na_2 EDTA complex in the presence of associated bacteria. This is important not only in studies of trace-element limitation (discussed in subsection 10.18) but also suggests the possible utilization of the nitrogen contained in the complex to support growth as well.

The standard addition of 1.00 mg Na_2 EDTA l^{-1} contains 0.075 mg N l^{-1}. If this nitrogen is bioavailable it would support an additional 2.85 ± 20% mg dry wt l^{-1} increase in *S. capricornutum* standing crop (0.075 x 38 = 2.85).

The additional response obtained with Na_2 EDTA addition over that in the control was 2.24 mg dry wt l^{-1}. Similar additional response (2.43 mg dry wt

l^{-1}) was obtained in the combination Na_2 EDTA plus phosphorus spiked test water (6.40 - 4.06).

These growth responses suggest that Na_2 EDTA may have been metabolized and that growth was obtained relative to its nitrogen content. This response also indicates the possible utilization of organic bound nitrogen fractions in the test waters as growth stimulators. Thus, the 1.5 fold increase in calculated bioavailable nitrogen may be due to organic nitrogen utilization rather than to unreliable TSIN chemical analysis.

The algal responses to this representative nitrogen limited test water were chosen to identify all of the possible nitrogen interactions that can regulate growth of *S. capricornutum* assayed in accordance with the prescribed test protocol. The metabolism of Na_2 EDTA and the subsequent utilization of its nitrogen content for support of additional growth has been defined in less than 1% of all nitrogen limited natural waters studied by this laboratory.

10.18 Trace-element limitation--Trace-element limitation is rare in most natural waters. Less than 2% of the 150 natural waters investigated by this laboratory were trace-element growth regulated. Growth in these trace-element limited waters was most often limited by the availability of iron.

Synthetic organic ligands such as Disodium (ethylenedinitrilo) tetraacetate (Na_2 EDTA) are added to defined inorganic culture media to make sure trace elements, principally Fe and Mn, are available to support algal growth. Recognition of the growth enhancement qualities of organic ligands led to addition of Na_2 EDTA to natural test waters prior to the assay to ascertain trace-element availability.

Columbia River water, collected at Rock Island Dam and Bridgeport, Washington, was identified as being trace-element deficient (Miller, Greene, Shiroyama, 1976a). The N:P ratios of these waters of 9:1 indicated potential nitrogen growth limitation, as did the theoretical yield predictions based on Ortho-P and TSIN content. The theoretical yield (±20%) for Columbia River water collected at Rock Island Dam based on TSIN content of 0.109 mg l^{-1} is 4.10 mg dry wt l^{-1} of the test alga. This water supported less than 10 percent of the predicted yield in the control, or in the control plus nitrogen or phosphorus added singly or in combination. The addition of 1.00 mg Na_2 EDTA l^{-1}, however, stimulated growth to 5.40 mg dry wt l^{-1}, 128 percent of the predicted control yield. The addition of Na_2 EDTA may have increased iron availability, thus stimulating growth. The concentrations of total soluble ferric iron that can be in equilibrium with ferric hydroxide at pH 8.0 in oxygenated water is approximately 0.2 µg l^{-1}. The iron requirement for optimum growth of *S. capricornutum* is 4.5 µg l^{-1}, 22.5 times greater than the normal concentration in soluble form. Addition of Na_2 EDTA stabilizes soluble iron availability in natural waters. Theis and Singer (1973) stated that the exact mechanism(s) by which organic ligands interact with iron are not known. Their research has shown that organic ligands, such as EDTA, can stabilize ferrous iron through the formation of organic complexes which are resistant to oxygenation in natural waters, thus increasing the availability of iron for aquatic growth. Barber (1973) studied growth enhancement effects of EDTA addition to sea water. He concluded that organic ligands may increase the mobility of essential metals such as Fe and Mn, but that these findings do not limit the possibility that organic ligands enhance phytoplankton growth by suppressing heavy metal toxicity. Without comprehensive trace-metal analysis no judgment may be made as to whether algal growth is limited by either suboptimal or toxic trace-metal content of the water (Miller, Greene, and Shiroyama, 1976b).

10.19 Nitrogen and phosphorus co-limitation--Nitrogen and phosphorus co-limitation is most commonly observed in high nutrient (eutrophic) waters. An analysis of the N:P ratios in these highly productive waters is usually sufficient to assess co-limitation conditions. N:P ratios ranging between 10 and 12:1 generally indicate possible co-limitation. Actual assay verification, using the nutrient and/or chelator additions outlined in section 10.12, is necessary to establish the nutrient limitation status of a test water. Growth response to the singular addition of nitrogen, phosphorus and Na_2 EDTA will be essentially identical (within ±20%) in N and P co-limited waters. Significant increase in growth response will only be obtained in the combined N and P, as well as in the N and P, and Na_2 EDTA combination, spiked test waters.

10.20 Practical application of nutrient limitation studies--Understanding of the interaction of nutrient dynamics and its regulation of aquatic productivity in natural waters is necessary to establish sound management alternatives.

No singular chemical test or biological measurement (i.e., Ortho-P or chlorophyll a) can be used to define all the interactions regulating biological productivity in natural waters. However, the "Algal Assay: Bottle Test" (AA:BT) can be used to define and/or predict the nutrient availability in most natural waters. This test can also identify and/or predict the algal growth potential of natural waters. For example: The AA:BT must be used to assess the trophic status of a natural water. In most cases the placement of a test water into a trophic category is based on the bioavailable nitrogen and phosphorus content of a test water. Those waters containing greater than 0.015 mg bioavailable P l^{-1} and 0.165 mg bioavailable N l^{-1} are considered eutrophic. In general, these values correlate to the Ortho-P and TSIN chemical analysis content in the test waters. Test waters in which the assay response does not correlate with their available N and P content may be trace-element growth regulated. As this is quite rare, toxicity is usually indicated in these test waters.

The AA:BT nutrient limitation experimental design growth responses can be used to define the effectiveness of the following management alternative:

> What is the effectiveness of an 80% reduction of domestic waste phosphorus loading upon receiving water quality?
>
> The AA:BT can verify the bioavailable phosphorus content of the wastewater; the post treatment available P content in the receiving water; and the interaction of other nutrients affecting biological productivity in the receiving water. For example: A domestic waste containing 7.5 mg Total P l^{-1} and 27.0 mg Total N l^{-1} is discharged into a receiving water. This treatment plant discharge contributes 60% of the total phosphorus and 40% of the total nitrogen to the receiving water. The downstream receiving water has an average total phosphorus content of 0.060 mg l^{-1}, 60% of which (0.036 mg l^{-1}) is due to the waste inflow. The remaining 40% phosphorus content (0.024 mg l^{-1}) is contributed upstream from the treatment plant.
>
> The assay response in the receiving water downstream from the facility outfall, before treatment of the domestic waste to reduce phosphorus loading, was 11.18 mg dry wt l^{-1}. The bioavailable phosphorus concen-

tration needed to support this yield is 0.026 mg P l^{-1}, (11.18 ÷ 430 = 0.026) which is 43% of the receiving water (0.060 mg l^{-1}) total phosphorus content.

The 80% reduction in total phosphorus loading should result in a final total phosphorus receiving water concentrations of 0.031 mg P l^{-1}.

$$[0.060 - 0.036 + (20\% \times 0.036) = 0.031]$$

where: 0.060 = mg l^{-1} downstream P concentration

0.036 = mg P l^{-1} contributed by treatment plant

20% × 0.036 = concentration of post treatment P l^{-1} contribution by treatment plant operating at 80% efficiency.

Assuming that a similar percentage (43%) of the post treatment receiving water total P is bioavailable, one would predict that a biomass of 5.78 mg dry wt l^{-1} (a reduction of 52%) would be obtained in the receiving water [430 (43%) × 0.031) = 5.78].

The AA:BT results suggest that:

(1) The 52% anticipated reduction in algal growth is still considered a eutrophic condition.

(2) A water use cost benefit analysis should be conducted before treatment is initiated.

(3) Phosphorus removal may only be necessary during peak growth conditions (July, August, September).

(4) Reduction of upstream phosphorus loading in conjunction with advanced wastewater treatment would vastly improve water quality.

Similar experimental design and analysis rationale can be used to define and help solve other water quality management problems such as:

(1) Determine the feasibility of nutrient criteria, i.e., establishing a 1.0 mg total P l^{-1} effluent standard.

(2) Define the "real world" impact of land use upon nutrient loading to receiving waters.

(3) Define and monitor the effectiveness of established effluent guidelines.

(4) Determine the effluent criteria for specific complex wastes based on their stimulatory or inhibitory properties.

10.30 Heavy metal toxicity

The ability of the "Algal Assay:Bottle Test" to predict the algal growth potential of lakes and streams and its use to define limiting nutrients in these natural waters led to the identification and application of nitrogen and phosphorus yield factors to predict the growth of S. capricornutum (section 10.10; Nutrient limitation).

Failure of a test water to attain the predicted yield or nutrient limitation (N, P, trace-element) status when assayed in accordance with the experimental design protocol outlined in subsection 10.12 usually indicates the presence of toxicants. The AA:BT can be used to define the interactions of heavy metals upon productivity within aquatic ecosystems.

The study of heavy metal interaction in natural waters is complicated by an uncertainty of the form, concentration, and biological reactive state of the metal. Thus, with few exceptions, the chemically analyzed heavy metal content of a test water may not reflect the resultant biological interactions and productivity in natural waters. The growth response of S. capricornutum to conditions of heavy metal stress in natural waters is in essence a "biological response model" of complex physical and chemical interactions. The resultant biological response (maximum standing crop) is an integration of the combined effects of solubility, ionic strength, metal concentration, and contact time which regulate toxicity of the heavy metal to the test organism.

10.31 Experimental design--The *basic experimental design* to determine the extent of heavy metal toxicity and its interaction upon nutrient regulation of the test alga in natural waters is shown in Table 10:

TABLE 10
BASIC EXPERIMENTAL DESIGN TO DEFINE HEAVY METAL TOXICITY

Control

Control + 1.00 mg Na_2 EDTA l^{-1}

Control + 1.00 mg Na_2 EDTA + 0.05 mg P l^{-1}

Control + 1.00 mg Na_2 EDTA + 1.00 mg N l^{-1}

Control + 1.00 mg Na_2 EDTA + 0.05 mg P and 1.00 mg N l^{-1}

10.32 Essential background data--The *minimum* chemical data necessary to substantiate the presence of heavy metal toxicity are: Initial pH; Total phosphorus; Ortho-P; Total Kjeldahl N; NO_2; NO_3 and NH_3-N. The growth response of the test algal is compared to the predicted yields based on the analyzed nutrient content of the test waste or receiving water. Those *wastes* or receiving waters *which do not support growth* within ± 20% of their limiting nutrient potential are then analyzed for the suspected heavy metals.

10.33 Test condition--Each test flask is inoculated to contain a final concentration of 1000 cells ml^{-1} of *S. capricornutum*; incubated at 24 ± 2°C under 4304 lumens (400 ± 10% ft-c) and shaken once daily (see section 4.21) or continuously for a period of at least 14 days.

10.34 Interpretation of results--*All* toxicity results *must* be reported as the *% inhibition* at day 14 (% I_{14}) based on the difference in mg dry wt l^{-1} obtained in the control with that produced in the Control test water containing 1.00 mg Na_2 EDTA l^{-1}.

The growth responses shown in Table 11A, and corresponding control test water nutrient chemical analysis data (Table 11B), are typical of those obtained in heavy metal contaminated receiving streams.

TABLE 11A
GROWTH RESPONSES REPRESENTATIVE OF HEAVY METAL TOXICITY

Sample Treatment	MSC (mg dry wt l^{-1})
Control	0.12 > 95% I_{14}
Control + 1.00 mg Na_2 EDTA l^{-1}	21.70
Control + 1.00 mg Na_2 EDTA + 0.05 mg P l^{-1}	20.90
Control + 1.00 mg Na_2 EDTA + 1.00 mg N l^{-1}	49.60
Control + 1.00 mg Na_2 EDTA + 1.00 mg N + 0.05 mg P l^{-1}	50.20

TABLE 11B
NUTRIENT ANALYSIS OF THE METAL CONTAMINATED CONTROL TEST
WATER AND CORRESPONDING PREDICTED YIELDS (mg l^{-1})

0.175 mg Total P l^{-1}

0.115 mg Ortho-P l^{-1} = 0.155 x 430 = 49.45 ± 20%

0.895 mg Total N l^{-1}

0.365 mg NO_3 + NO_2-N l^{-1}

0.144 mg NH_3-N l^{-1}

0.509 mg TSIN l^{-1} = 0.509 x 38 = 19.30 ± 20%

4.4:1 N:P ratio (TSIN ÷ Ortho-P)

The > 95% I_{14} growth response obtained in this test water is indicative of heavy metal toxicity. The addition of 1.00 mg Na_2 EDTA l^{-1} to this test water complexed the bioreactive metals, enabling the test alga to achieve the maximum nitrogen limited standing crop of 21.70 mg dry wt l^{-1}. The 2.2 fold increase in maximum yield, beyond that achieved in the chelated control, obtained with combined chelator and nitrogen addition suggests that nitrogen is the secondary growth-regulating constituent. The addition of phosphorus to this nitrogen, chelator combination did not stimulate growth greater than that predicted for the TSIN content (1.509 x 38 = 57.34 ± 20%) of the test water.

The inhibited growth response obtained in the control test water is attributed to its analyzed heavy metal content. This test water contained 0.125 mg Zn l^{-1}, 0.006 mg Cu l^{-1}, 0.001 mg Cd l^{-1}, 0.038 mg Al l^{-1} and 0.009 mg Pb l^{-1}.

These growth responses have established the sensitivity of _S. capricornutum_ to the bioreactive state of these heavy metals. The >95% I_{14} algistatic response of the test alga in the control test water is similar to that of sensitive indigenous species to accidental or recent discharges of heavy metals (an algicidal response is verified when a subculture from an algistatic test water fails to grow in assay medium). However, this inhibited response does not necessarily reflect the growth potential of indigenous algae which have evolved from long term chronic exposure to heavy metals.

The response of the standard laboratory algal test organism to the addition of Na_2 EDTA, singly and in combination with nitrogen and phosphorus, to heavy metal laden test waters has been shown to correlate (r = 0.82) with indigenous phytoplankton standing crop (Greene _et al._, 1978). The indigenous phytoplankton growth in these waters can be attributed to: (1) adaptation to their environment; (2) natural decomposition and/or complexing of the heavy metals by both organic and inorganic ligands; and (3) the presence of adequate nutrients.

10.40 New product evaluation

10.41 Introduction--The advent of recent Toxic Substance Control legislation outlined in Public Law 94-469; (October 11, 1976) and the urgent need to establish test procedures and effluent guidelines for pollutants has led to a flurry of bioassessment activity. This activity is relevant because only the bioreactive components of the pollutants are responsible for the regulation of biological productivity in natural waters.

The continued acceptance of chemical analysis of specific constituents within the product formulations (i.e., Zn, Cu, Cd, phenol, PCB, aniline) as the primary reference standard for the legislation of ecological response criteria is both unwise and misleading. Only concurrent evaluation of both chemical analysis and bioassay results will provide the scientific base necessary to establish realistic water quality criteria.

The AA:BT can be used to define the potential stimulatory and/or inhibitory properties of new product formulations introduced into receiving waters.

10.42 Experimental design--It is important to consider the following factors when designing an assay experiment to evaluate the environmental impact of new product formulations:

(1) The geographical distribution and intended use of the product.

(2) The method of entry into the receiving water (i.e., direct discharge or discharge after primary, secondary or advanced wastewater treatment, etc.).

(3) The recommended application formulation.

(4) The anticipated final concentrations (usage and dilution ratios) of the product within the receiving water.

(5) The degree to which the test waters are representative of those within the geographical area of product use.

The relative importance of these factors will vary with each specific product that is evaluated. However, basic experimental design criteria and rationale are applicable in evaluating all new product formulations.

The following experimental protocol is an example of how the AA:BT is used to define the potential stimulatory and/or inhibitory impact of new detergent formulations. The impact of detergent formulations upon aquatic productivity is most often ascribed to the product's nutrient (primarily phosphorus) content. Detergent derived-nutrients usually enter receiving waters as components of domestic waste water effluents. Procter and Gamble (1976) estimate that 35% of the phosphates in domestic sewage originates from detergents.

The amount of a candidate detergent formulated product to be added to a test water can be calculated directly from historical treatment plant phosphorus loading curves (i.e., 35% of the phosphorus in sewage x % of waste loading to receiving water) or from theoretical detergent loading equations (Hall, 1973).

A sample detergent loading calculation based on 12,000 wash loads per day, treated and discharged from a treatment plant (15×10^6 liters per day) into a receiving water containing 854×10^6 liters per day (349 cfs) is as follows:

Assume:

(1) one cup of detergent (73×10^3 mg) added to each washload.

(2) 30% of the detergent remains after waste treatment.

Therefore: $\frac{A \times B \times C}{D} \times E = F$

where; A = mg detergent per wash load.

B = no. of washloads per day (population ÷ 3.26 = washloads per day).*

C = % detergent remaining after treatment.

D = liters per day of waste discharge (mgd x 3.79)

E = % treated waste contained in receiving water (liters per day waste discharge ÷ liters per day in receiving water). Note! cfs x 2.448 = 1×10^6 liters per day.

F = mg l^{-1} of detergent in receiving water.

$$\frac{73 \times 10^3 \times 12{,}000 \times 0.30}{15 \times 10^6 \text{ liters}} \times .017 = 0.30 \text{ mg } l^{-1}$$

Thus, in this example, 0.30 mg l^{-1} of detergent would be contained in the receiving water mixing zone downstream from the domestic waste discharge.

Receiving waters must be used in the evaluation of new product formulations. These test waters should be collected upstream from the waste inflow in accordance with the methods outlined in section 3.1.

A typical receiving water assessment should include the calculated product concentration as well as 0.5 and 5.0 fold (mg l^{-1}) additions, or other additions as deemed environmentally significant.

A minimum assay evaluation of the test detergent in our example would include:

(1) Control receiving water(s)

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* After Hall, 1973.

(2) Control + 0.15 mg detergent l^{-1}

(3) Control + 0.30 mg detergent l^{-1}

(4) Control + 1.50 mg detergent l^{-1}

Assay results obtained from this experimental design will determine the effect of the material above, but not the effect of the material in addition to or its interaction with the current wastewater discharge to the receiving stream. These effects can be obtained by expanding the experimental design to include; (1) upstream receiving water plus the % wastewater equal to the normal background level, and (2) wastewater plus the predetermined detergent levels.

The degree of growth stimulation or inhibition of the test material added to a receiving water is usually defined by dividing the 14-day mg dry wt l^{-1} MSC in the treated test water by the MSC supported in the control test water. In some test waters the MSC may not be achieved until after day 14 (see criteria outlined in section 8.3). Maximum standing crop assessment in these waters should be made at 2-day intervals following day 14 until the maximum yield is obtained.

Treated:Control ratios < 1.0 indicate inhibition, while ratios > 1.0 suggest stimulation. Stimulation ratios are expressed as statements of the receiving water product concentration responsible for the stimulation at the time of maximum yield (e.g., 0.31 mg detergent l^{-1} = 2.8).

Inhibition responses can be reported as either the % inhibition at the time in days the MSC is obtained (e.g., $\%I_{14}$) based upon the difference in mg dry wt l^{-1} obtained in the control water with that produced in the treated water, or as the aforementioned Treated:Control ratio, i.e., 0.5. Inhibition can be either algistatic or algicidal. The subculture of the test alga from an apparent algistatic test water, into the algal assay medium (section 5.0), must be used to verify an algicidal response.

Reduction of 50% in MSC (EC_{50}) is not an acceptable toxic response for assessment of algal growth. Miller, Greene, and Shiroyama (1976), have reported that the inhibition of specific heavy metals upon the growth of S. capricornutum may be linear (0 - 100%) with the increase in zinc content of test waters, but non-linear for the increase in copper and cadmium content beyond 20 and 40% respectively. Additional increase in either Cu of Cd resulted in > 95% I_{14} of the test alga. Payne and Hall (1978), also discourage the use of EC_{50} response values to define the toxic effects of new detergent formulations.

10.5 Evaluation of Complex Wastes

10.51 Introduction--Point and/or non-point waste effluents generated from industrial, agricultural, and domestic treatment and sludge disposal activities usually contain both inorganic and organic components. The interaction of these complex wastes and the extent to which they regulate biological productivity in natural waters is not well defined. This is in part due to past research in which the response of selected test organisms to specific constituents (i.e., Zn, Cd, Cr, Cu, DDT, PCB, etc.) cultured in defined media was used to establish toxicity criteria. This concept is faulty because it does not reflect the antagonistic and/or synergistic interactions of the organic and inorganic ligands contained within both the complex wastes and the receiving waters. This shortcoming, coupled with the use of chemical analysis data as the basis for biological water quality criteria, has caused concern among regulating agencies.

The AA:BT is centered around the concept that only the bioreactive components of pollutants are responsible for the regulation of biological productivity in natural waters. Therefore, the bioassay should be used to screen the inhibitory and/or stimulatory properties of the complex wastes before an expensive chemical analysis regime is initiated. This approach is useful because the bioreactive components of the waste will be identified. Those wastes which are inhibitory would be analyzed for their toxic components, while those that stimulate productivity would not.

This approach is beneficial for at least two reasons: (1) It eliminates unnecessary expense of organic and/or heavy metal analysis; and (2) the assay results are usually obtained before the chemical data are available for evaluation.

Twenty-three textile waste samples, representative of eight manufacturing processes, were evaluated by seven assay techniques to define their toxic properties. The bioassessment organisms included freshwater and marine algae, crustacae, fish and mammals (Rawlings, 1978). A comparison of the sensitivity of these bioassays (Table 12) showed that the AA:BT, using *S. capricornutum*, was the most sensitive test used in the textile waste survey. This test not only identified the toxic wastes, it also identified those that were stimulatory.

Forty-three percent (10 of 23) of the wastes surveyed were inhibitory and the remaining 57% wastes were stimulatory (Shiroyama *et al*. in preparation).

Chemical analysis of the organic and heavy metal content of these textile wastes was initiated at the time of collection. A savings of $19,500 could have been realized if the AA:BT had been used to screen the wastes prior to

TABLE 12
COMPARATIVE BIOTEST RESPONSES FOR TEXTILE EFFLUENTS*†

	Freshwater ecology series			Recommended interpretation		Marine ecology series		
Textile Plant	Fathead minnow (96-hr LC_{50}), % secondary effluent	*Daphnia* (48-hr EC_{50}), % secondary effluent	*Selenastrum* (14-day EC_{50}), % secondary effluent	*Selenastrum* 20% secondary effluent % I_{14}	% S_{14}	Sheepshead minnow (96-hr LC_{50}), % secondary effluent	Grass shrimp (96-hr LC_{50}), % secondary effluent	Algae (96-hr EC_{50}), % secondary effluent
A	19.0	9.0	11.3	53	----	62.0	21.2	f
B	NAT[b]	NAT	----	--	83	NAT	NAT	g
C	46.5	41.0	----	--	187	69.5	12.8	90
D	NAT	NAT	----	--	100	f	f	f
E	NAT	7.8	< 2.0	95[e]	----	NAT	NAT	10 to 50
F	NAT	81.7	----	--	598	NAT	NAT	85
G	64.7	62.4	----	--	390	NAT	NAT	59
H	c	40% dead at 100% concentration	7.8	92	----	f	f	f
J	NAT	NAT	----	--	76	f	f	f
K	NAT	NAT	----	--	57	NAT	NAT	77
L	23.5	28.0	12.0	81	--	NAT	NAT	1.7
M	NAT	60.0	----	--	149	f	f	f
N	48.8	100% dead at all dilutions	< 2.0	95[e]	--	47.5	26.3	2.3
P[a]	NAT	NAT	----	--	38	f	f	9.0
R	16.5	8.0	8.8	95	--	f	f	f
S	NAT	NSA[d]	----	--	382	NAT	NAT	g
T	46.5	NAT	----	--	1911	68.0	34.5	70
U	NAT	12.1	----	--	377	NAT	NAT	g
V	36.0	9.4	----	--	232	f	f	94
W	55.2	6.3	1.0	95	--	37.5	19.6	50
X	NAT	NAT	----	--	163	NAT	NAT	g
Y	NAT	NAT	----	--	261	f	f	f
Z	NAT	42.6	15.5	84	--	f	f	f

[a] Sample inadvertently collected prior to settling pond. [b] No acute toxicity. [c] Diseased batch of fish nullified this analysis. [d] No statistical analysis because heavy solids concentration obscured the analysis; the sample did not appear to be acutely toxic. [e] 95% growth inhibition in 2% solution of secondary effluent. [f] Analysis not performed on this sample. [g] Growth inhibition < 50% in 100% solution of secondary effluent. [*] No chemical mutagen was detected by the 10 microbial strains. [†] No rat mortality after 14 days due to maximum dosage of 10^{-5} m^3/kg body weight (LD_{50}). However, six samples (B, C, F, L, N, and S) showed potential body weight effects, and sample R resulted in eye irritation.

chemical analysis. This savings is based on the $1500 cost per analysis spent for each of the thirteen stimulatory wastes.

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11.0 APPENDICES

11.1 Bibliography--The following references cite research using Selenastrum species to study the effects of nutrients, toxicants, complex wastes, and specific inorganic and organic compounds upon algal productivity. These citations are indicative of the importance of algal assays in the study and management of water quality problems. Reprints of omitted or current research citations should be sent to Mr. William E. Miller (address on title page) for publication in future addenda. Because of Xerox regulations, reprints other than our own are not available from the Corvallis Environmental Research Laboratory.

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11.2 Recommended equipment and supplies--The AA:BT is an economical test which can provide information not attainable by any other method. An average cost of analysis for a comprehensive study, e.g., basic evaluation of a complex waste effluent, is approximately $400.00. This consists of the evaluation of four waste concentrations compared to the control receiving water, comprising a total of five tests × three replicates or fifteen test flasks. This cost includes basic chemical anlaysis for TSIN and Ortho-P as well as capitalization and operation expenses. In general each test, consisting of three replicate flasks, costs approximately $80.00. As mentioned earlier, the AA:BT should be used to screen pollutants before establishing extensive physical, chemical and biological monitoring programs. The savings obtained will more than pay for the initial cost of $15,000 to establish the capability to adequately perform routine assay analysis. The following is a cost breakdown of the necessary supplies and equipment:

1. FLASKS, Erlenmeyer, narrow mouth, heavy-duty top. (KIMAX, PYREX)*
   500 ml -------------------------- 36/case ----- @$37.00 (1978)
2. FLASKS, Erlenmeyer, wide mouth. (KIMEX, PYREX)
   125 ml -------------------------- 48/case ----- @$44.00 (1978)
3. FOAM TUBE PLUGS--Gaymar IDENTI-PLUGS. (Vendor: VWR)
   200 ct --- @$22.00 (1978)
   Order by number L800-C, size fits opening 27 to 34 mm
   Manufactured by GAYMAR INDUSTRIES, INC., ONE BANK ST.,
   ORCHARD PARK, NY 14127
   (Cheaper foam plugs are available--you may run into
   toxicity problem with the cheaper ones; therefore check
   for toxicity before using them.)
4. LIGHTS, (3) 40 w. fluorescent fixtures with (6) "cool white"
   lamps and light bank frame ---------------------- @$85.00 (1978)
5. GYROTORY SHAKER, w/o platform. (LAB-LINE, NEW BRUNSWICK
   SCIENTIFIC) ------------------------------------ @$950.00 (1978)
   (Better to make your own platforms, see
   schematic Figure 6.)

SHAKER PLATFORM SCHEMATIC

# Req.	*List of Materials*	
	3/4" A-C Exterior Plywood	
1	20 3/4" x 45 1/4" slotted on 4 3/32" centers, slots 3/16" deep, 3/16" wide, to give 4" x 4" interior dimension compartment; 4 slots lengthwise, 10 slots crosswise, to yield 55 compartments.	
	3/16" Masonite or Equivalent	
4	3/4" x 45 1/4" strip	glued in
10	3/4" x 20 3/4" strip	glued in
	1" x 2" (Nominal) Clear Fir or Equivalent	
2	22" strip, mitered ends	
2	46 3/4" strip, mitered ends	

Finish with 2 coats flat white paint (latex exterior house paint acceptable).

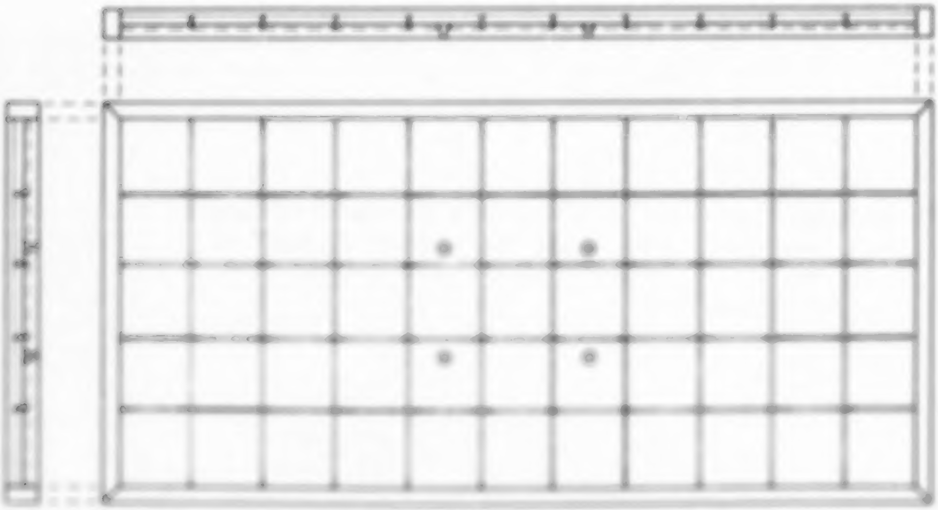

Figure 4. Schematic and list of materials for 500 ml Erlenmeyer flask shaker platform.

6. TEST TUBE RACK OR SUPPORT, vinyl coated. Holds 40 tubes -- @$8.00 (1978)
(5/8" diameter and 7 7/8" L × 3 1/2" W × 3 1/4" H)

7. BELLCO BEAKER, Modified for coulter counter (BELLCO)
12/box -- @$25.00 (1978)
BELLCO GLASS, INC.
340 EDRUDO RD
VINELAND, NJ 08380 Tel: 609/691-1075

8. MICRO PIPETTING SYSTEM. 1 ml w/o tips. (OXFORD,
EPPENDORF) ------------------------------------ @$49.00- (1978
$65.00
Disposable tips -------------------- 1000/pk -- @$45.00- (1978)
$55.00

9. PIPETTOR. 1.0 to 10.0 ml dispenser. (OXFORD, REPIPET,
UNIVERSAL) ------------------------------------ @$80.00- (1978)
$160.00

10. MILLIPORE MEMBRANE
A. 0.45 μm, 47 mm diameter plain, autoclaved
pack or sterile pack ----- 100/pk --------- @$24.00 (1977)
0.22 μm, 47 mm diameter, plain, sterile
100/pk ------------------------------------ @$24.00 (1977)

B. Millipore funnel hydrosol stainless 47 mm- @$186.30 (1977)
OR
PYREX 47 mm Glass Funnel ------------------ @$44.80 (1977)
Teflon-faced Pyrex 47 mm funnel ----- @$55.00 (1977)
MILLIPORE CORP.
BEDFORD, MASS 01730 Tel: 800/225-1380,
in Mass., (617)275-9200

11. COULTER COUNTER ZBI, w/ 70 & 100 μ aperature tube ------- @$8000.00 (1978)
MCV/HCT Flatpack to go with ZBI --------------- @$3500.00 (1978)
COULTER ELECTRONICS, INC.
590 WEST 20TH ST
HIALEAH, FL 33010

12. HEAT EXCHANGER -----------cost depends on room size and
number of light banks and shakers as well as
ability of facility to maintain temperature
within 20°C.

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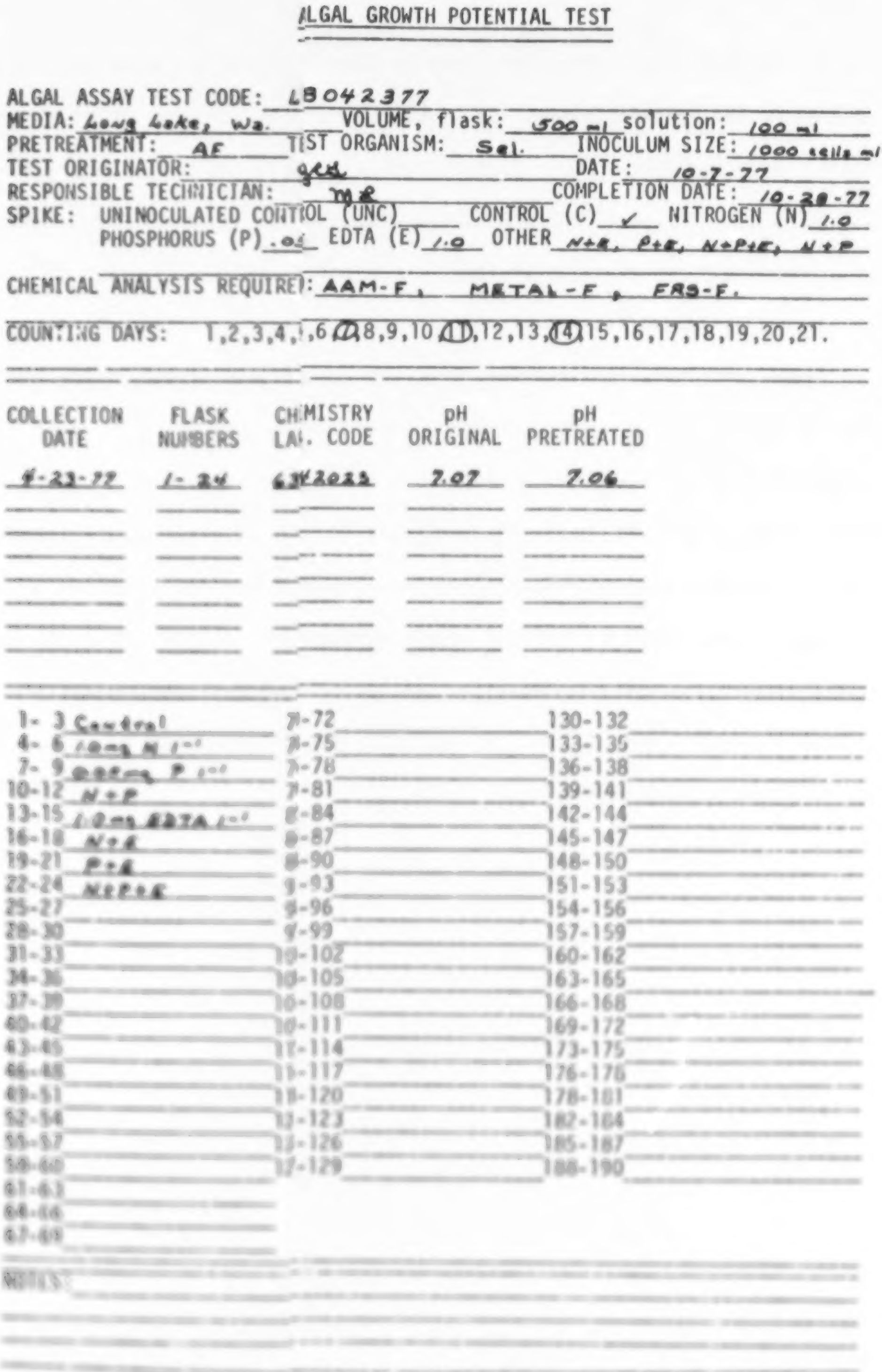
* Mention of Trade names or commercial products and sources does not constitute endorsement by the U.S. Environmental Protection Agency.

11.3 FORTRAN data reduction program--The algal assay data analysis system consists of two programs: (1) ALGASSY (pages 89-94), which reads the data cards and produces a line printer summary and a data file which is input to; (2) ASSYPLOT (pages 95-98), which produces plots of mean dry weight versus time. These programs written in FORTRAN IV are currently running on the CDC 3300 operated by the Miline-Computer Center, Oregon State University. Listings of these programs and sample input and output are provided for analysis and use in establishing a similar data reduction format.

Completed data reduction formats for assays conducted on a test water collected from Long Lake, Washington (pages 84-88) are included as examples of data reduction used to facilitate computer enumeration and plotting of assay data. Note: Line 0010 in ALGASSY program (page 89) is the inclusion of the older dry weight yield conversion factor (2.0×10^{-7}) as determined in section 8.4. This factor is now 3.6×10^{-7} and may differ according to values obtained by each investigator.



ALGAL GROWTH POTENTIAL TEST

ALGAL ASSAY TEST CODE: LB042377
MEDIA: Long Lake, Wa. VOLUME, flask: 500 ml solution: 100 ml
PRETREATMENT: AF TEST ORGANISM: Sel. INOCULUM SIZE: 1000 cells ml^{-1}
TEST ORIGINATOR: gcd DATE: 10-7-77
RESPONSIBLE TECHNICIAN: mr COMPLETION DATE: 10-28-77
SPIKE: UNINOCULATED CONTROL (UNC) ____ CONTROL (C) ✓ NITROGEN (N) 1.0
PHOSPHORUS (P) .05 EDTA (E) 1.0 OTHER N+E, P+E, N+P+E, N+P

CHEMICAL ANALYSIS REQUIRED: AAM-F, METAL-F, FRS-F.

COUNTING DAYS: 1,2,3,4,5,6,⑦,8,9,10,⑪,12,13,⑭,15,16,17,18,19,20,21.

COLLECTION DATE	FLASK NUMBERS	CHEMISTRY LAB. CODE	pH ORIGINAL	pH PRETREATED
4-23-77	1-24	63W2025	7.07	7.06

1- 3	Control	70-72		130-132	
4- 6	1.0mg N l^{-1}	73-75		133-135	
7- 9	0.05mg P l^{-1}	76-78		136-138	
10-12	N+P	79-81		139-141	
13-15	1.0mg EDTA l^{-1}	82-84		142-144	
16-18	N+E	85-87		145-147	
19-21	P+E	88-90		148-150	
22-24	N+P+E	91-93		151-153	
25-27		94-96		154-156	
28-30		97-99		157-159	
31-33		100-102		160-162	
34-36		103-105		163-165	
37-39		106-108		166-168	
40-42		109-111		169-172	
43-45		112-114		173-175	
46-48		115-117		176-178	
49-51		118-120		178-181	
52-54		121-123		182-184	
55-57		124-126		185-187	
58-60		127-129		188-190	
61-63					
64-66					
67-69					

NOTES:

Figure 5. Completed algl growth potential test design format.

Date Sampled April 23, 1977

TEST CODE: LB 042377

MEDIA: AF Long Lake, Wa.

SPIKE: C, 1.0mg N l^{-1}, 0.05mg P l^{-1}, 1.0mg EDTA l^{-1}
N+P, N+E, P+E, N+P+E

SPIKE RANGE: – –

TEST VOLUME: 100 ml

CONTAINER VOLUME: 500 ml

# REPLICATE FLASKS: 3

STOCK CULTURE DATA:

MEDIA: 100% AAM

DAYS GROWTH: 7

INOCULUM:

Selenastrum Alga

1004 cells/ml

0.011 mg/l Dry Wt.

56 MCV

TEST PREPARATION:

TEST WATERS: ML

DISPENSED: ML

SPIKED: ML

INOCULUM: ML

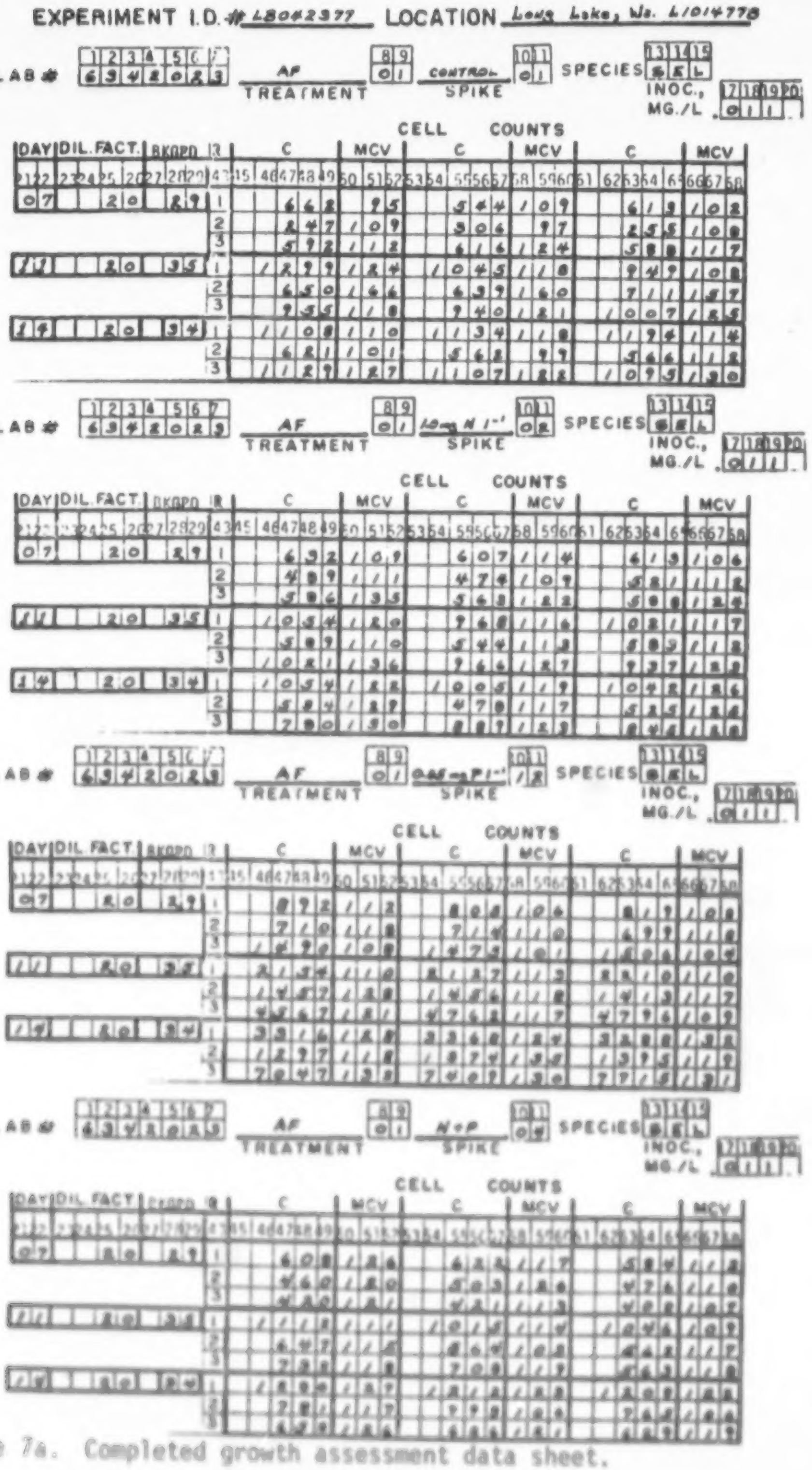
INOCULATED: ML

SAMPLED: ML

COUNTED: ML

FRESH SPIKE:

Figure 6. Completed growth assessment cover sheet



EXPERIMENT I.D. # L80#2377 LOCATION Long Lake, Wa. L1014778

LAB # 6342023 (1–7) AF TREATMENT 01 (8 9) CONTROL SPIKE 01 (10 11) SPECIES SEL (13 14 15) INOC., MG./L .011 (17 18 19 20)

CELL COUNTS

DAY	DIL. FACT.	BKGRD	R	C	MCV	C	MCV	C	MCV
21 22	23 24 25 26	27 28 29	43	45 46 47 48 49	50 51 52	53 54 55 56 57	58 59 60	61 62 63 64 65	66 67 68
07	20	29	1	662	95	544	109	619	102
			2	247	109	304	97	255	108
			3	592	112	616	124	588	117
11	20	35	1	1299	124	1045	118	949	108
			2	650	146	639	160	711	157
			3	955	118	940	121	1007	125
14	20	34	1	1108	110	1134	118	1194	114
			2	681	101	568	99	566	112
			3	1129	127	1107	128	1095	130

LAB # 6342023 (1–7) AF TREATMENT 01 (8 9) 10 mg N l⁻¹ SPIKE 02 (10 11) SPECIES SEL (13 14 15) INOC., MG./L .011 (17 18 19 20)

CELL COUNTS

DAY	DIL. FACT.	BKGRD	R	C	MCV	C	MCV	C	MCV
21 22	23 24 25 26	27 28 29	43	45 46 47 48 49	50 51 52	53 54 55 56 57	58 59 60	61 62 63 64 65	66 67 68
07	20	29	1	632	109	607	114	613	106
			2	489	111	474	109	581	112
			3	586	135	563	122	588	124
11	20	35	1	1054	120	968	116	1081	117
			2	589	110	544	113	583	118
			3	1021	136	966	127	937	128
14	20	34	1	1054	122	1005	119	1042	126
			2	584	129	478	117	525	124
			3	780	130	887	123	848	128

LAB # 6342023 (1–7) AF TREATMENT 01 (8 9) 0.05 mg P l⁻¹ SPIKE 12 (10 11) SPECIES SEL (13 14 15) INOC., MG./L .011 (17 18 19 20)

CELL COUNTS

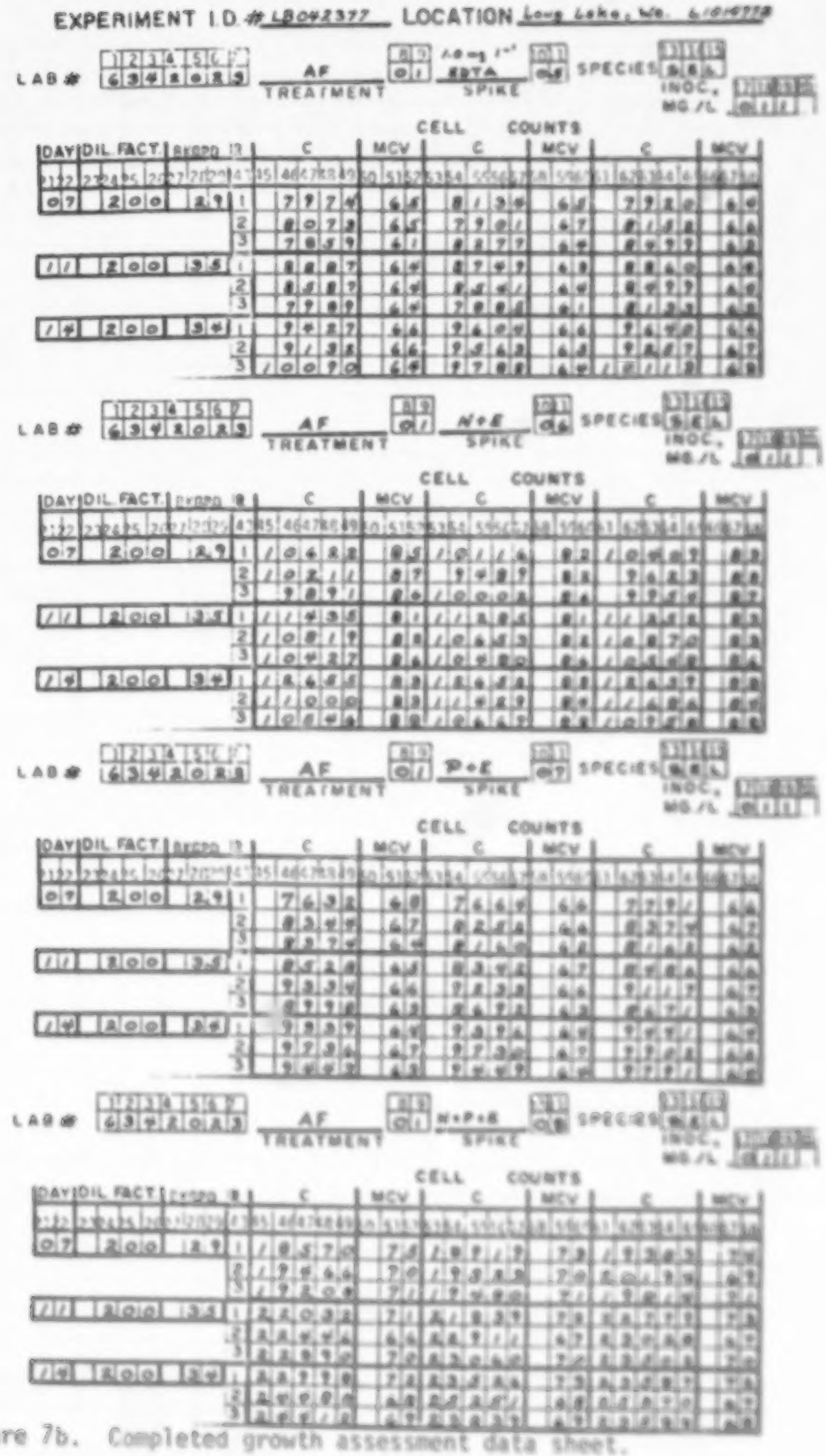
DAY	DIL. FACT.	BKGRD	R	C	MCV	C	MCV	C	MCV
21 22	23 24 25 26	27 28 29	43	45 46 47 48 49	50 51 52	53 54 55 56 57	58 59 60	61 62 63 64 65	66 67 68
07	20	29	1	892	112	808	106	819	108
			2	710	118	714	110	499	118
			3	1490	108	1479	101	1604	104
11	20	35	1	2154	110	2127	113	2210	110
			2	1457	128	1456	118	1413	117
			3	4267	121	4768	117	4796	109
14	20	34	1	3516	126	3368	124	3288	132
			2	1297	118	1874	132	1395	119
			3	7047	138	7409	130	7715	131

LAB # 6342023 (1–7) AF TREATMENT 01 (8 9) N+P SPIKE 04 (10 11) SPECIES SEL (13 14 15) INOC., MG./L .011 (17 18 19 20)

CELL COUNTS

DAY	DIL. FACT.	BKGRD	R	C	MCV	C	MCV	C	MCV
21 22	23 24 25 26	27 28 29	43	45 46 47 48 49	50 51 52	53 54 55 56 57	58 59 60	61 62 63 64 65	66 67 68
07	20	29	1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
			2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
			3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
11	20	35	1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
			2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
			3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
14	20	34	1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
			2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
			3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Figure 7a. Completed growth assessment data sheet.



EXPERIMENT I.D. # LB042377 LOCATION Long Lake, Wa.

LAB # 6342025 AF TREATMENT 01 1.0 mg l⁻¹ EDTA SPIKE 05 SPECIES INOC. MG./L

CELL COUNTS

DAY 07 DIL. FACT. 200 BKGRD 29
DAY 11 DIL. FACT. 200 BKGRD 35
DAY 14 DIL. FACT. 200 BKGRD 34

LAB # 6342025 AF TREATMENT 01 N+E SPIKE 06 SPECIES INOC. MG./L

CELL COUNTS

DAY 07 DIL. FACT. 200 BKGRD 29
DAY 11 DIL. FACT. 200 BKGRD 35
DAY 14 DIL. FACT. 200 BKGRD 34

LAB # 6342025 AF TREATMENT 01 P+E SPIKE 07 SPECIES INOC. MG./L

CELL COUNTS

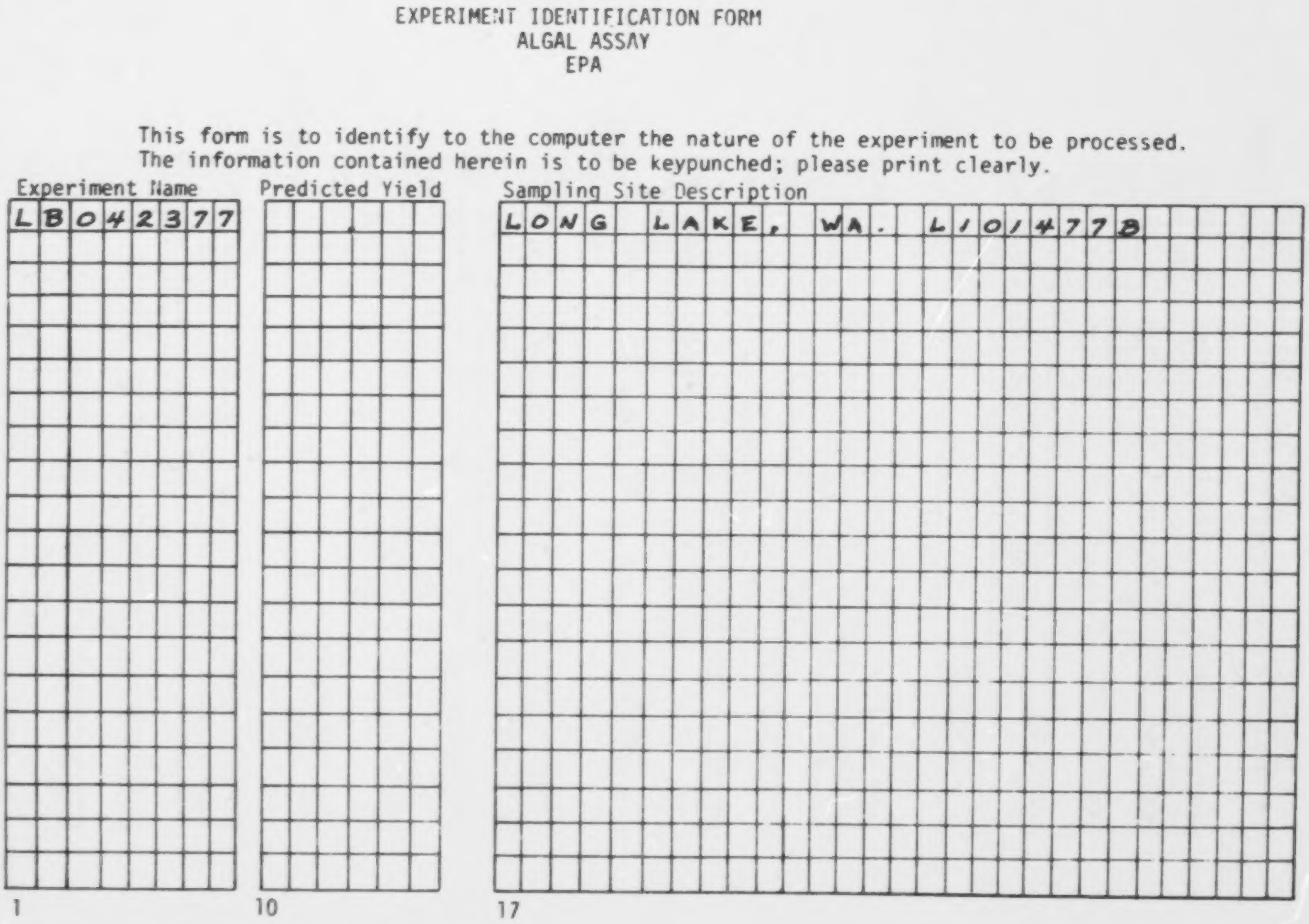
DAY 07 DIL. FACT. 200 BKGRD 29
DAY 11 DIL. FACT. 200 BKGRD 35
DAY 14 DIL. FACT. 200 BKGRD 34

LAB # 6342025 AF TREATMENT 01 N+P+E SPIKE 08 SPECIES INOC. MG./L

CELL COUNTS

DAY 07 DIL. FACT. 200 BKGRD 29
DAY 11 DIL. FACT. 200 BKGRD 35
DAY 14 DIL. FACT. 200 BKGRD 34

Figure 7b. Completed growth assessment data sheet.



EXPERIMENT IDENTIFICATION FORM
ALGAL ASSAY
EPA

This form is to identify to the computer the nature of the experiment to be processed. The information contained herein is to be keypunched; please print clearly.

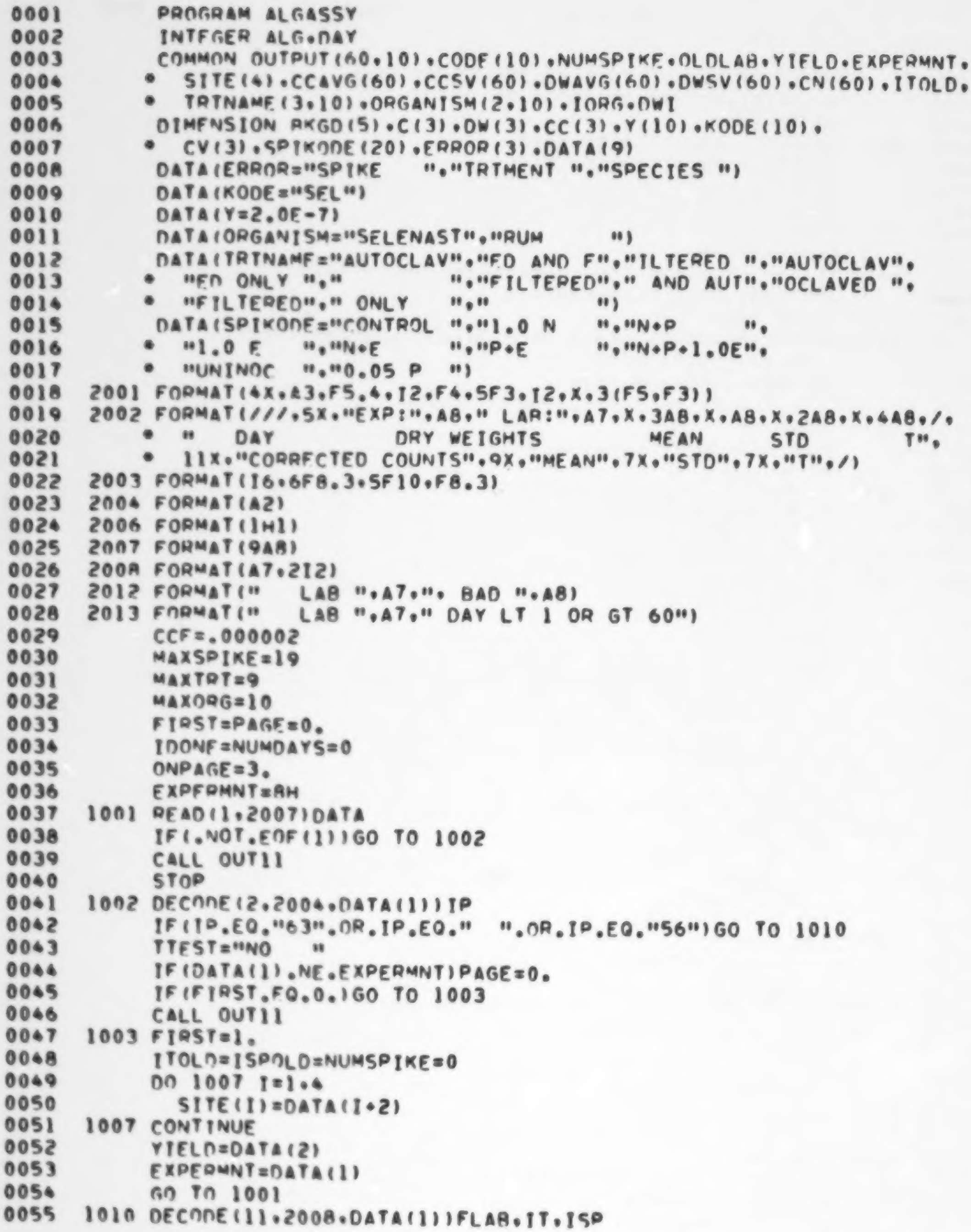
Experiment Name: LB042377

Predicted Yield: .

Sampling Site Description: LONG LAKE, WA. L/0/477B

1 10 17

Figure 8. Completed Computer ID format.



```
      PROGRAM ALGASSY
      INTEGER ALG,DAY
      COMMON OUTPUT(60,10),CODE(10),NUMSPIKE,OLDLAB,YIELD,EXPERMNT,
     *  SITE(4),CCAVG(60),CCSV(60),DWAVG(60),DWSV(60),CN(60),ITOLD,
     *  TRTNAME(3,10),ORGANISM(2,10),IORG,DWI
      DIMENSION BKGD(5),C(3),DW(3),CC(3),Y(10),KODE(10),
     *  CV(3),SPIKODE(20),ERROR(3),DATA(9)
      DATA(ERROR="SPIKE   ","TRTMENT ","SPECIES ")
      DATA(KODE="SEL")
      DATA(Y=2.0E-7)
      DATA(ORGANISM="SELENAST","RUM     ")
      DATA(TRTNAME="AUTOCLAV","ED AND F","ILTERED ","AUTOCLAV",
     *  "ED ONLY ","           ","FILTERED"," AND AUT","OCLAVED ",
     *  "FILTERED"," ONLY   ","        ")
      DATA(SPIKODE="CONTROL ","1.0 N   ","N+P     ",
     *  "1.0 E   ","N+E     ","P+E     ","N+P+1.0E",
     *  "UNINOC  ","0.05 P  ")
 2001 FORMAT(4X,A3,F5.4,I2,F4,5F3,I2,X,3(F5,F3))
 2002 FORMAT(///,5X,"EXP:",A8," LAB:",A7,X,3A8,X,A8,X,2A8,X,4A8,/,
     *  "   DAY           DRY WEIGHTS          MEAN      STD        T",
     *  11X,"CORRECTED COUNTS",9X,"MEAN",7X,"STD",7X,"T",/)
 2003 FORMAT(I6,6F8.3,5F10,F8.3)
 2004 FORMAT(A2)
 2006 FORMAT(1H1)
 2007 FORMAT(9A8)
 2008 FORMAT(A7,2I2)
 2012 FORMAT("   LAB ",A7,", BAD ",A8)
 2013 FORMAT("   LAB ",A7," DAY LT 1 OR GT 60")
      CCF=.000002
      MAXSPIKE=19
      MAXTRT=9
      MAXORG=10
      FIRST=PAGE=0.
      IDONE=NUMDAYS=0
      ONPAGE=3.
      EXPERMNT=8H
 1001 READ(1,2007)DATA
      IF(.NOT.EOF(1))GO TO 1002
      CALL OUT11
      STOP
 1002 DECODE(2,2004,DATA(1))IP
      IF(IP.EQ."63".OR.IP.EQ."  ".OR.IP.EQ."56")GO TO 1010
      TTEST="NO    "
      IF(DATA(1).NE.EXPERMNT)PAGE=0.
      IF(FIRST.EQ.0.)GO TO 1003
      CALL OUT11
 1003 FIRST=1.
      ITOLD=ISPOLD=NUMSPIKE=0
      DO 1007 I=1,4
        SITE(I)=DATA(I+2)
 1007 CONTINUE
      YIELD=DATA(2)
      EXPERMNT=DATA(1)
      GO TO 1001
 1010 DECODE(11,2008,DATA(1))FLAB,IT,ISP
```

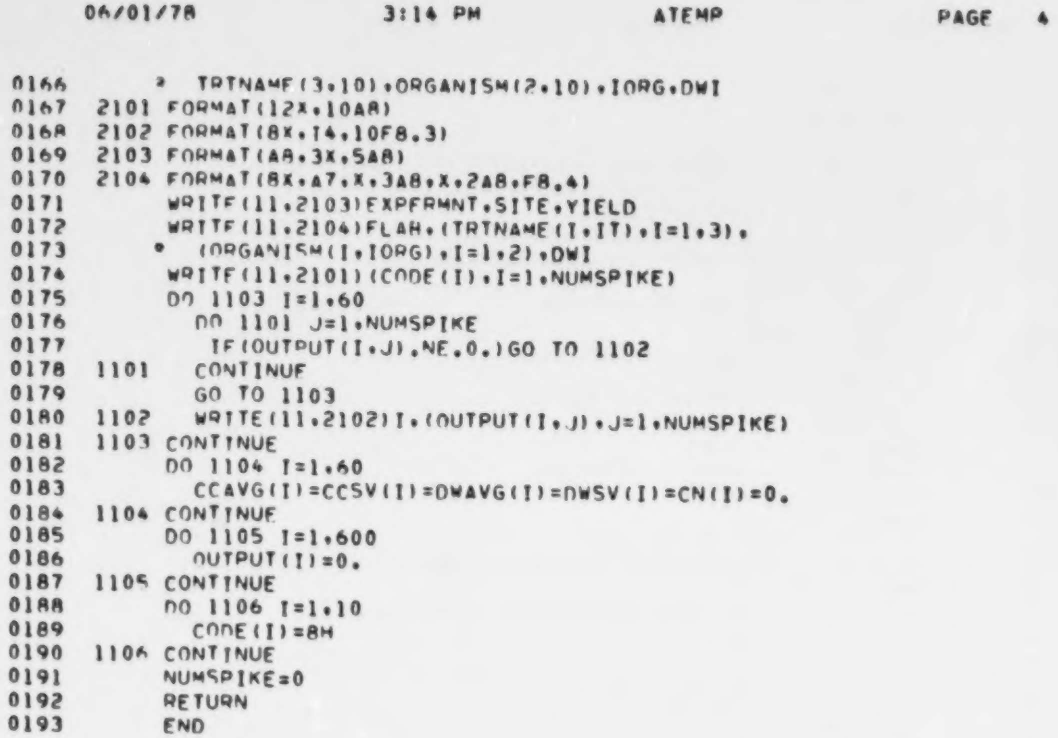
Figure 9a. ALGASSY, computer program data reduction printout.

```
06/01/78                3:14 PM                ATEMP                PAGE   2

      IF(IT.GT.0.AND.IT.LE.MAXTRT)GO TO 1011
      WRITE(61,2012)FLAB,ERROR(2)
      GO TO 1001
 1011 IF(ITOLD.EQ.IT)GO TO 1012
      IF(ITOLD.NE.0)CALL OUT11
      PAGE=0.
      ITOLD=IT
      ISPOLD=0
      OLDLAB=FLAB
 1012 IF(ISP.GT.0.AND.ISP.LE.MAXSPIKE)GO TO 1013
      WRITE(61,2012)FLAB,ERROR(1)
      GO TO 1001
 1013 DECODE(60,2001,DATA(2))ALG,DWI,DAY,D,BKGD,IREP,
     *  (C(I),CV(I),I=1,3)
      DO 1014 IORG=1,MAXORG
        IF(ALG.EQ.KODE(IORG))GO TO 1015
 1014 CONTINUE
      WRITE(61,2012)FLAB,ERROR(3)
      GO TO 1001
 1015 IF(DAY.GE.1.AND.DAY.LE.60)GO TO 1016
      WRITE(61,2013)FLAB
      GO TO 1001
C  DATA HAS PASSED CHECKS
 1016 IF(ISPOLD.EQ.ISP)GO TO 1020
      NUMSPIKE=NUMSPIKE+1
      CODE(NUMSPIKE)=SPIKODE(ISP)
      ISPOLD=ISP
      IF(PAGE.EQ.0.)WRITE(31,2006)
      WRITE(31,2002)EXPERMNT,FLAB,(TRTNAME(I,IT),I=1,3),SPIKODE(ISP),
     *  (ORGANISM(I,IORG),I=1,2),SITE
      IF(IDONE.NE.1)GO TO 1019
      IF(NUMDAYS.LE.9)ONPAGE=4.
 1019 IDONE=IDONE+1
      PAGE=PAGE+1.
      IF(PAGE.GE.ONPAGE)PAGE=0.
 1020 TIMES=SUM=0.
      DO 1021 I=1,5
        IF(BKGD(I).EQ.0.)GO TO 1021
        SUM=SUM+BKGD(I)
        TIMES=TIMES+1.
 1021 CONTINUE
      IX=IFIX(SUM/TIMES+.5)
      X=FLOAT(IX)
C  CALCULATE DRY WEIGHT AND CORRECTED COUNTS
      CSUM=CVSUM=COUNTS=0.
      DO 1022 I=1,3
        IF(C(I).EQ.0.)GO TO 1022
        COUNTS=COUNTS+1.
        CSUM=CSUM+C(I)
        CVSUM=CVSUM+CV(I)
 1022 CONTINUE
      IF(COUNTS.LE.0.)GO TO 1023
      AVGC=CSUM/COUNTS
      AVGCV=CVSUM/COUNTS
      DW(IREP)=.0001
```

Figure 9a

```
      CC(IREP)=.4
      DIFF=AVGC-X
      IF(DIFF.LE.0.)GO TO 1023
      DW(IREP)=D*Y(IORG)*DIFF*(1.+CCF*DIFF)*AVGCV
      CC(IREP)=D*DIFF*(1.+CCF*DIFF)
 1023 IF(IREP.LT.3)GO TO 1001
C  CALCULATE MEAN AND STANDARD DEVIATION
      CCSUM=CCSUMSQ=DWSUM=DWSUMSQ=REPS=0.
      DO 1024 I=1,3
        IF(DW(I).NE.0.)REPS=REPS+1.
        DWSUM=DWSUM+DW(I)
        DWSUMSQ=DWSUMSQ+DW(I)*DW(I)
        CCSUM=CCSUM+CC(I)
        CCSUMSQ=CCSUMSQ+CC(I)*CC(I)
 1024 CONTINUE
      DWMEAN=DWSUM/REPS
      OUTPUT(DAY,NUMSPIKE)=DWMEAN
      CCMEAN=CCSUM/REPS
      DWSTD=CCSTD=0.
      IF(REPS.LT.2.)GO TO 1025
      SVDW=(DWSUMSQ-DWSUM*DWSUM/REPS)/(REPS-1.)
      DWSTD=SQRT(SVDW)
      SVCC=(CCSUMSQ-CCSUM*CCSUM/REPS)/(REPS-1.)
      CCSTD=SQRT(SVCC)
 1025 TCC=TDW=0.
      IF(ISP.NE.1)GO TO 1026
      IF(REPS.LT.2)GO TO 1026
      TTEST="YES  "
C  SAVE CONTROL DATA FOR T TEST
      DWAVG(DAY)=DWMEAN
      DWSV(DAY)=SVDW
      CCAVG(DAY)=CCMEAN
      CCSV(DAY)=SVCC
      CN(DAY)=REPS
      GO TO 1027
C  COMPUTE T STATISTIC
 1026 IF(TTEST.EQ."NO   ")GO TO 1027
      IF(CN(DAY).LT.2..OR.REPS.LT.2.)GO TO 1027
      DDW2=(CN(DAY)+REPS)*((CN(DAY)-1.)*DWSV(DAY)+(REPS-1.)*SVDW)
     *  /CN(DAY)/REPS/(CN(DAY)+REPS-2.)
      TDW=ABSF(DWAVG(DAY)-DWMEAN)/SQRT(DDW2)
      DCC2=(CN(DAY)+REPS)*((CN(DAY)-1.)*CCSV(DAY)+(REPS-1.)*SVCC)
     *  /CN(DAY)/REPS/(CN(DAY)+REPS-2.)
      TCC=ABSF(CCAVG(DAY)-CCMEAN)/SQRT(DCC2)
 1027 WRITE(31,2003)DAY,DW,DWMEAN,DWSTD,TDW,CC,CCMEAN,CCSTD,TCC
      IF(IDONE.EQ.1)NUMDAYS=NUMDAYS+1
      DO 1028 I=1,3
        DW(I)=CC(I)=0.
 1028 CONTINUE
      GO TO 1001
      END

      SUBROUTINE OUT11
      COMMON OUTPUT(60,10),CODE(10),NUMSPIKE,FLAB,YIELD,EXPERMNT,
     *  SITE(4),CCAVG(60),CCSV(60),DWAVG(60),DWSV(60),CN(60),IT,
```

Figure 9a



```
06/01/78              3:14 PM              ATEMP                  PAGE   4

        *  TRTNAME(3,10),ORGANISM(2,10),IORG,DWI
 2101 FORMAT(12X,10A8)
 2102 FORMAT(8X,I4,10F8.3)
 2103 FORMAT(A8,3X,5A8)
 2104 FORMAT(8X,A7,X,3A8,X,2A8,F8.4)
      WRITE(11,2103)EXPERMNT,SITE,YIELD
      WRITE(11,2104)FLAH,(TRTNAME(I,IT),I=1,3),
     *  (ORGANISM(I,IORG),I=1,2),DWI
      WRITE(11,2101)(CODE(I),I=1,NUMSPIKE)
      DO 1103 I=1,60
        DO 1101 J=1,NUMSPIKE
          IF(OUTPUT(I,J).NE.0.)GO TO 1102
 1101   CONTINUE
        GO TO 1103
 1102   WRITE(11,2102)I,(OUTPUT(I,J),J=1,NUMSPIKE)
 1103 CONTINUE
      DO 1104 I=1,60
        CCAVG(I)=CCSV(I)=DWAVG(I)=DWSV(I)=CN(I)=0.
 1104 CONTINUE
      DO 1105 I=1,600
        OUTPUT(I)=0.
 1105 CONTINUE
      DO 1106 I=1,10
        CODE(I)=8H
 1106 CONTINUE
      NUMSPIKE=0
      RETURN
      END
```

Figure 9a

```
  EXP:LB042377 LAB:6342023 AUTOCLAVED AND FILTERED  CONTROL  SELENASTRUM      LONG LAKE, WA L1014778
DAY          DRY WEIGHTS          MEAN      STD         T                CORRECTED COUNTS          MEAN       STD          T

   7     .236      .101      .268      .202      .089         0       11560      4809     11406      9258      3854         0
  11     .497      .407      .453      .453      .045         0       21299     12649     18681     17543      4436         0
  14     .508      .229      .545      .427      .173         0       22276     10992     21573     18280      6322         0


  EXP:LB042377 LAB:6342023 AUTOCLAVED AND FILTERED  1.0 N    SELENASTRUM      LONG LAKE, WA L1014778
DAY          DRY WEIGHTS          MEAN      STD         T                CORRECTED COUNTS          MEAN       STD          T

   7     .258      .206      .280      .248      .038      .833       11781      9322     11012     10705      1258      .618
  11     .462      .240      .485      .395      .135      .694       19625     10752     18829     16402      4909      .299
  14     .490      .237      .409      .379      .129      .389       20033      9576     16106     15238      5282      .640


  EXP:LB042377 LAB:6342023 AUTOCLAVED AND FILTERED  0.05 P   SELENASTRUM      LONG LAKE, WA L1014778
DAY          DRY WEIGHTS          MEAN      STD         T                CORRECTED COUNTS          MEAN       STD          T

   7     .353      .314      .612      .426      .162     2.101       16220     13592     29312     19708      8421     1.954
  11     .949      .683     2.182     1.271      .800     1.770       42755     28219     94340     55105     34748     1.857
  14    1.696      .657     3.911     2.088     1.662     1.721       66233     26497    149291     80674     62658     1.716


  EXP:LB042377 LAB:6342023 AUTOCLAVED AND FILTERED  N+P      SELENASTRUM      LONG LAKE, WA L1014778
DAY          DRY WEIGHTS          MEAN      STD         T                CORRECTED COUNTS          MEAN       STD          T

   7     .273      .214      .176      .221      .049      .332       11527      9021      7753      9434      1920      .070
  11     .456      .249      .300      .335      .108     1.739       20495     11132     12669     14766      5021      .718
  14     .596      .328      .294      .406      .166      .154       24044     14936     12048     17009      6261      .247
```

Figure 9b. ALGASSY, computer program reduction printout.

```
  EXP:LB042377 LAB:6342023 AUTOCLAVED AND FILTERED  1.0 E     SELENASTRUM       LONG LAKE, WA L1014778
DAY           DRY WEIGHTS            MEAN      STD         T               CORRECTED COUNTS            MEAN        STD         T

   7  20.972  21.493  20.847  21.104    .343 102.205   1621541   1628283   1663316   1637713     22427 123.949
  11  22.797  22.149  20.290  21.745   1.302  28.317   1790355   1730417   1618858   1713210     87034  33.702
  14  25.620  24.963  25.878  25.487    .472  86.392   1940875   1891139   2032305   1954773     71602  46.662


  EXP:LB042377 LAB:6342023 AUTOCLAVED AND FILTERED  N.E       SELENASTRUM       LONG LAKE, WA L1014778
DAY           DRY WEIGHTS            MEAN      STD         T               CORRECTED COUNTS            MEAN        STD         T

   7  35.226  33.383  34.937  34.515    .991  59.712   2113543   1987055   2023363   2041320     65128  53.947
  11  37.710  36.150  36.699  36.853    .791  79.531   2308777   2195321   2133681   2212593     88816  42.753
  14  42.937  38.804  38.432  40.058   2.501  27.384   2586585   2318950   2183641   2363059    205061  19.796


  EXP:LB042377 LAB:6342023 AUTOCLAVED AND FILTERED  P.E       SELENASTRUM       LONG LAKE, WA L1014778
DAY           DRY WEIGHTS            MEAN      STD         T               CORRECTED COUNTS            MEAN        STD         T

   7  20.758  22.485  20.900  21.381    .959  38.091   1556844   1686385   1667516   1636915     69982  40.223
  11  22.595  24.841  22.441  23.292   1.343  29.437   1711738   1872404   1781039   1788394     80585  38.004
  14  24.405  26.522  24.724  25.217   1.141  37.200   1906629   1989133   1941705   1945823     41406  79.707


  EXP:LB042377 LAB:6342023 AUTOCLAVED AND FILTERED  N.P.E     SELENASTRUM       LONG LAKE, WA L1014778
DAY           DRY WEIGHTS            MEAN      STD         T               CORRECTED COUNTS            MEAN        STD         T

   7  58.149  57.056  57.453  57.553    .553 177.323   3928979   4094948   4045992   4023306     85278  81.444
  11  66.717  63.456  67.716  65.963   2.228  50.920   4633144   4759207   4836867   4743073    102815  79.533
  14  70.671  71.049  68.820  70.180   1.193 100.245   4885100   5249927   5011187   5048738    185289  46.996
```

Figure 9b

```
     05/26/78               1:45 PM              ASSYPLOT              PAGE   1

           PROGRAM ASSYPLOT
           DIMENSION LX(11),LY(7),DW(10),DATA(12),ORGANISM(2),XB(2),
          *  YB(2),IHT(3),ISP(10),YLABEL(3),MRK(10),SITE(4),DAYS(20),
          *  POINTS(20,10),SPIKODE(10),IN(20),XBIAS(2)
           DATA(XBIAS=0.,15.)
           DATA(XB=0.,24.)
           DATA(YB=-3.,3.)
           DATA(LX="0 ","4 ","8 ","12","16","20","24","28","32","36","40")
           DATA(LY="-3","-2","-1","0 ","1 ","2 ","3 ")
           DATA(YLABEL="M G  D R"," Y  W T "," L      ")
           DATA(MRK=2,4,18,20,22,28,10,12,14,16)
      2001 FORMAT(12A8)
      2002 FORMAT(3X,4A8,F8.3)
      2003 FORMAT(8X,A7,X,3A8,X,2A8,F8.4)
      2004 FORMAT(12X,10A8)
      2005 FORMAT(F4,10F8.3)
      2006 FORMAT(F4," PLOTS PRODUCED")
           CALL TK4010
           CALL PLOTTYPE(0)
           ICROSS=1
           CALL SIZE(29.,12.)
           IDW="I"
           IPRE="P"
           DAYLABEL="D A Y S"
           TEN="10"
           ISTOP=4HNO
           PLOTS=0.
           CALL ERASE
      1001 READ(1,2001)DATA
           IF(.NOT.EOF(1))GO TO 1002
           ISTOP=4HYES
           GO TO 1003
      1002 IF(DATA(1).EQ.8H        )GO TO 1022
           IF(PLOTS.EQ.0.)GO TO 1020
     C  DRAW HORIZONTAL BOUNDARIES
      1003 LIM=24
           NX=7
           XB(2)=24.
           IF(DAYLAST.LT.24.)GO TO 1004
           ID=IFIX(DAYLAST+.1)
           IF(ID.GT.39)ID=39
           NX=2+ID/4
           LIM=4*(NX-1)
           XB(2)=FLOAT(LIM)
      1004 CALL SCALE(.25,1.5,XBIAS(ICROSS),0.,-6.,-4.)
           DO 1006 IB=1,2
             CALL PLOT(0.,YB(IB),0,0)
             DO 1005 J=1,LIM
               X=FLOAT(J)
               MARK=7
               MULT=J/4
               IP=J-4*MULT
               IF(IP.EQ.0)MARK=8
               IF(J.EQ.LIM)MARK=0
               CALL PLOT(X,YB(IB),1,MARK)
```

Figure 10

```
 1005   CONTINUE
 1006 CONTINUE
C  LABEL X AXIS
      CALL PLOT(-.2,-3.2,0,0)
      X=-.2
      DO 1007 I=1,NX
        CALL SYMBOL(X,-3.2,0.,.16,2,LX(I))
        X=X+4.
 1007 CONTINUE
      CALL SYMBOL(9.5,-3.5,0.,.16,7,DAYLABEL)
      CALL SYMBOL(0.,3.1,0.,.16,8,EXPERMNT)
      CALL SYMBOL(8.,3.1,0.,.16,29,SITE)
C  DRAW VERTICAL BOUNDARIES
      DO 1010 IB=1,2
        CALL PLOT(XB(IB),-3.,0,0)
        Y=.001
        DO 1009 IDEC=1,6
          DY=Y
          DO 1008 I=1,8
            Y=Y+DY
            EXP=ALOG10(Y)
            CALL PLOT(XB(IB),EXP,1,5)
 1008     CONTINUE
          Y=Y+DY
          EXP=ALOG10(Y)
          MARK=6
          IF(IDEC.EQ.6)MARK=0
          CALL PLOT(XB(IB),EXP,1,MARK)
 1009   CONTINUE
 1010 CONTINUE
C  LABEL Y AXIS
      TENY=-3.05
      EXPY=-2.9
      DO 1011 I=1,7
        CALL SYMBOL(-2.,TENY,0.,.16,2,TEN)
        CALL SYMBOL(-.8,EXPY,0.,.08,2,LY(I))
        TENY=TENY+1.
        EXPY=EXPY+1.
 1011 CONTINUE
      CALL SYMBOL(-3.,-1.,90.,.16,18,YLABEL)
      CALL SYMBOL(-3.4,1.15,90.,.08,2,LY(3))
      CALL SYMBOL(-5.,-3.,90.,.16,16,ORGANISM)
      CALL SYMBOL(-5.,-1.,90.,.16,24,TRT)
      CALL SYMBOL(-5.,2.,90.,.16,7,FLAB)
C  PLOT INOCULUM CONCENTRATION AND PREDICTED YIELD
      IF(DWI.LT..001.OR.DWI.GE.1000.)GO TO 1012
      YINOC=ALOG10(DWI)
      CALL SYMBOL(.2,YINOC,0.,.08,1,IDW)
 1012 IF(YIELD.LT..001.OR.YIELD.GE.1000.)GO TO 1013
      X=XB(2)-1.
      Y=ALOG10(YIELD)
      CALL SYMBOL(X,Y,0.,.08,1,IPRE)
C  PLOT POINTS
 1013 DO 1015 I=1,NSPK
        CALL PLOT(.5,YINOC,0,0)
```

Figure 10

```
05/26/78            1:45 PM              ASSYPLOT              PAGE   3

        DO 1014 N=1,NDAY
          IF(POINTS(N,I).GT.1000..OR.POINTS(N,I).LT..001)GO TO 1014
          Y=ALOG10(POINTS(N,I))
          CALL PLOT(DAYS(N),Y,1,MRK(I))
 1014   CONTINUE
 1015 CONTINUE
C  WRITE LEGEND
      XM=1.
      XN=2.
      Y=2.7
      DO 1016 I=1,NSPK
        CALL PLOT(XM,Y,0,MRK(I))
        CALL PLOT(XM,Y,1,MRK(I))
        CALL SYMBOL(XN,Y,0.,.16,8,SPIKODE(I))
        Y=Y-.2
 1016 CONTINUE
      IF(ISTOP.EQ.4HNO  )GO TO 1017
      CALL PLOTEND
      WRITE(61,2006)PLOTS
      STOP
 1017 ICROSS=ICROSS+1
      IF(ICROSS.LE.2)GO TO 1020
      ICROSS=1
      CALL TEKPAUSE
      CALL ERASE
C  DECODE DATA
 1020 EXPERMNT=DATA(1)
      PLOTS=PLOTS+1.
      DECODE(43,2002,DATA(2))SITE,YIELD
      READ(1,2003)FLAB,TRT,ORGANISM,DWI
      READ(1,2004)SPIKODE
      NSPK=NDAY=0
      DO 1021 I=1,10
       IF(SPIKODE(I).EQ.8H        )GO TO 1021
       NSPK=NSPK+1
 1021 CONTINUE
      GO TO 1001
 1022 DECODE(84,2005,DATA(2))DAY,DW
      NDAY=NDAY+1
      DAYS(NDAY)=DAY
      DO 1023 I=1,NSPK
        POINTS(NDAY,I)=DW(I)
 1023 CONTINUE
      DAYLAST=DAY
      GO TO 1001
      END
```

Figure 10

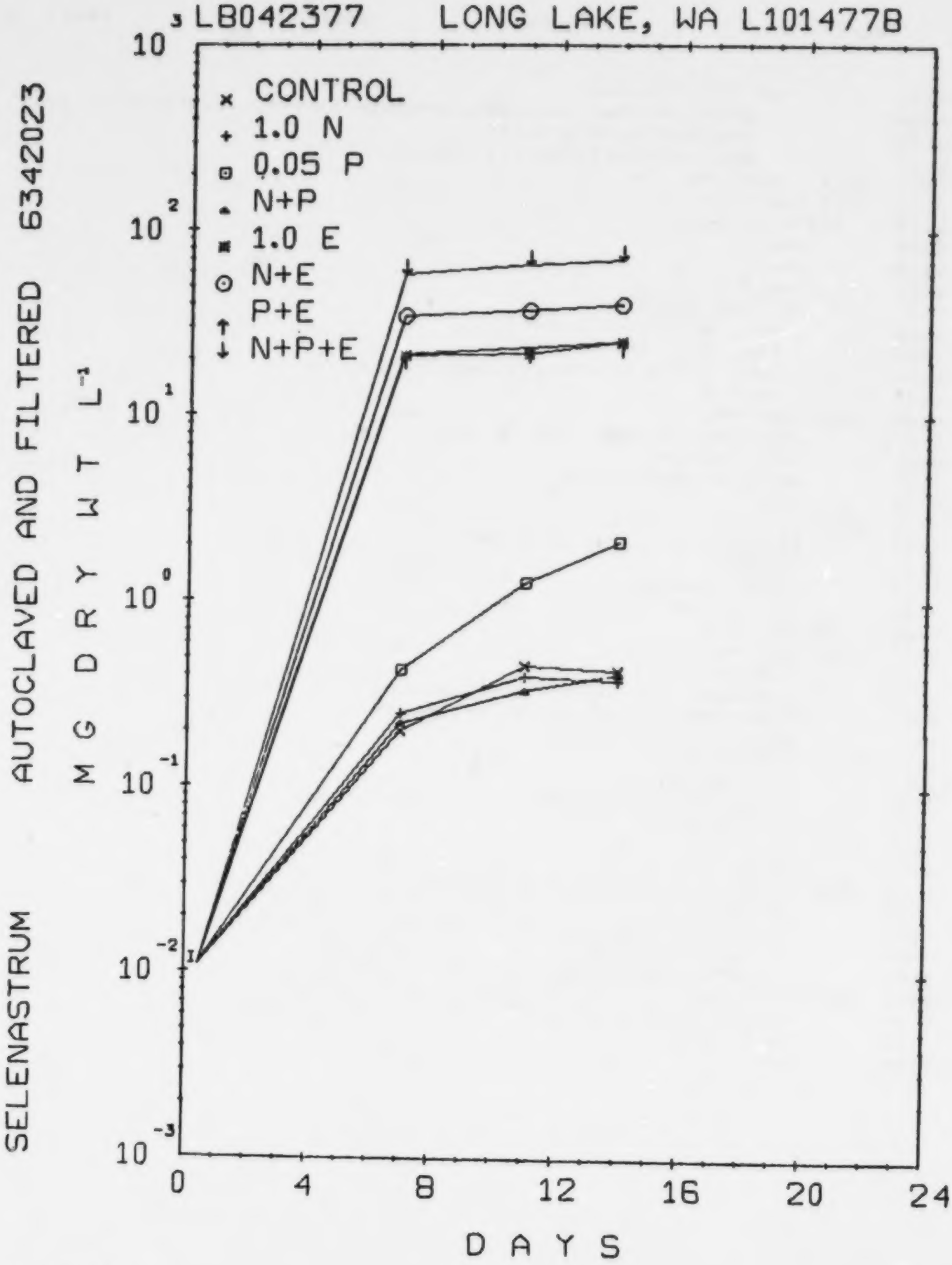

LB042377 LONG LAKE, WA L101477B
SELENASTRUM AUTOCLAVED AND FILTERED 6342023
MG DRY WT L⁻¹
CONTROL
1.0 N
0.05 P
N+P
1.0 E
N+E
P+E
N+P+E
10³
10²
10¹
10⁰
10⁻¹
10⁻²
10⁻³
0
4
8
12
16
20
24
DAYS

Figure 10

11.4 Standard algal assay forms--The following data reduction and experimental design formats are intended as a guide to facilitate laboratory identification and data analysis for test waters which are assayed.

Figure 11. Algal growth potential test design format.

ALGAL GROWTH POTENTIAL TEST

ALGAL ASSAY TEST CODE:

MEDIA: VOLUME, flask: solution:

PRETREATMENT: TEST ORGANISM: INOCULUM SIZE:

TEST ORIGINATOR: DATE:

RESPONSIBLE TECHNICIAN: COMPLETION DATE:

SPIKE: UNINOCULATED CONTROL (UNC) CONTROL (C) NITROGEN (N)
PHOSPHORUS (P) EDTA (E) OTHER

CHEMICAL ANALYSIS REQUIRED:

COUNTING DAYS: 1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21.

COLLECTION DATE	FLASK NUMBERS	CHEMISTRY LAB. CODE	pH ORIGINAL	pH PRETREATED

1- 3
4- 6
7- 9
10-12
13-15
16-18
19-21
22-24
25-27
28-30
31-33
34-36
37-39
40-42
43-45
46-48
49-51
52-54
55-57
58-60
61-63
64-66
67-69
70-72
73-75
76-78
79-81
82-84
85-87
88-90
91-93
94-96
97-99
100-102
103-105
106-108
109-111
112-114
115-117
118-120
121-123
124-126
127-129
130-132
133-135
136-138
139-141
142-144
145-147
148-150
151-153
154-156
157-159
160-162
163-165
166-168
169-172
173-175
176-178
178-181
182-184
185-187
188-190

NOTES:

Figure 12. Dilution test design format.

DILUTION TEST DESIGN

ALGAL ASSAY TEST CODE:__________ CHEM. LAB. CODE__________

TEST ELEMENT OR COMPOUND:__________

SPIKES:__________

MEDIA:__________ VOLUME, flask__________ solution__________

COLLECTION OR PREPARATION DATE:__________ PRETREATMENT:__________

TEST ORGANISM:__________ INOCULUM SIZE:__________

COUNTING DAYS: 1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,
23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41.

CHEMICAL ANALYSIS REQUIRED:__________

TEST ORIGINATOR:__________ DATE:__________

RESPONSIBLE TECHNICIAN__________ COMPLETION DATE__________

1- 3					61- 63				
4- 6					64- 66				
7- 9					67- 69				
10-12					70- 72				
13-15					73- 75				
16-18					76- 78				
19-21					79- 81				
22-24					82- 84				
25-27					85- 87				
28-30					88- 90				
31-33					91- 93				
34-36					94- 96				
37-39					97- 99				
40-42					100-102				
43-45					103-105				
46-48					106-108				
49-51					109-111				
52-54					112-114				
55-57					115-117				
58-60					118-120				

NOTES OR SPECIAL INSTRUCTIONS:__________

Figure 13. Dose/response test design format.

DOSE/RESPONSE TEST DESIGN

ALGAL ASSAY TEST CODE:____________ CHEM. LAB. CODE____________

TEST ELEMENT OR COMPOUND:____________

SPIKES:____________

MEDIA:____________ VOLUME, flask________ solution________

COLLECTION OR PREPARATION DATE:____________ PRETREATMENT:____________

TEST ORGANISM:____________ INOCULUM SIZE:____________

COUNTING DAYS: 1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,
23,24,25,26,27,28,29,30.31,32,33,34,35,36,37,38,39,40,41.

CHEMICAL ANALYSIS REQUIRED:____________

TEST ORIGINATOR:____________ DATE:____________

RESPONSIBLE TECHNICIAN____________ COMPLETION DATE____________

1- 3					61- 63				
4- 6					64- 66				
7- 9					67- 69				
10-12					70- 72				
13-15					73- 75				
16-18					76- 78				
19-21					79- 81				
22-24					82- 84				
25-27					85- 87				
28-30					88- 90				
31-33					91- 93				
34-36					94- 96				
37-39					97- 99				
40-42					100-102				
43-45					103-105				
46-48					106-108				
49-51					109-111				
52-54					112-114				
55-57					115-117				
58-60					118-120				

NOTES OR SPECIAL INSTRUCTIONS:____________

Figure 14. Growth assessment data cover sheet.

Date Sampled____________

TEST CODE: ____________________

MEDIA: ____________________

SPIKE: ____________________

SPIKE RANGE: ____________________

TEST VOLUME: ____________________

CONTAINER VOLUME: ________________

# REPLICATE FLASKS: ______________

STOCK CULTURE DATA:

MEDIA: __________

DAYS GROWTH: _____

Inoculum:

______________Alga

______________cells/ml

______________mg/l Dry Wt.

______________MCV

TEST PREPARATION

TEST WATERS: ____________________

DISPENSED: ____________________

SPIKED: ____________________

INOCULM: ____________________

INOCULATED: ____________________

SAMPLED: ____________________

COUNTED: ____________________

FRESH SPIKE: ____________________

Figure 15. Growth assessment data sheet (short form).

LOCATION ____________ EXP. I.D. ____________ START DATE ____________

TEST FLASK NUMBERS ____________

1 2 3 4 5 6 7	______	8 9	______	10 11	13 14 15	INOC., 17 18 19 20
LAB #	TREATMENT		SPIKE		SPECIES	MG/L

DAY	DILUTION FACTOR	BACKGROUND	R	COUNTS	MCV	COUNTS	MCV	COUNTS	MCV	CALC. DRY WEIGHT
21 22	23 24 25 26	27 28 29	43	45 46 47 48 49	50 51 52	53 54 55 56 57	58 59 60	61 62 63 64 65	66 67 68	
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							

1 2 3 4 5 6 7	______	8 9	______	10 11	13 14 15	INOC., 17 18 19 20
LAB #	TREATMENT		SPIKE		SPECIES	MG/L

DAY	DILUTION FACTOR	BACKGROUND	R	COUNTS	MCV	COUNTS	MCV	COUNTS	MCV	CALC. DRY WEIGHT
21 22	23 24 25 26	27 28 29	43	45 46 47 48 49	50 51 52	53 54 55 56 57	58 59 60	61 62 63 64 65	66 67 68	
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							

Figure 16. Growth assessment data sheet (long form).

LOCATION ______________ EXP. I.D. ______________ START DATE __________

TEST FLASK NUMBERS ______________

1 2 3 4 5 6 7	______	8 9	______	10 11	13 14 15	INOC., 17 18 19 20
LAB #	TREAT-MENT		SPIKE		SPECIES	MG/L

DAY	DILUTION FACTOR	BACK-GROUND	R	COUNTS	MCV	COUNTS	MCV	COUNTS	MCV	CALC. DRY WEIGHT
21 22	23 24 25 26	27 28 29	43	45 46 47 48 49	50 51 52	53 54 55 56 57	58 59 60	61 62 63 64 65	66 67 68	
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							
			1							______
			2							
			3							

Figure 17. Computer ID format.

EXPERIMENT IDENTIFICATION FORM
ALGAL ASSAY
EPA

This form is to identify to the computer the nature of the experiment to be processed. The information contained herein is to be keypunched; please print clearly.

Experiment Name

1

Predicted Yield

10

Sampling Site Description

17

Figure 18. Dry weight versus time format.

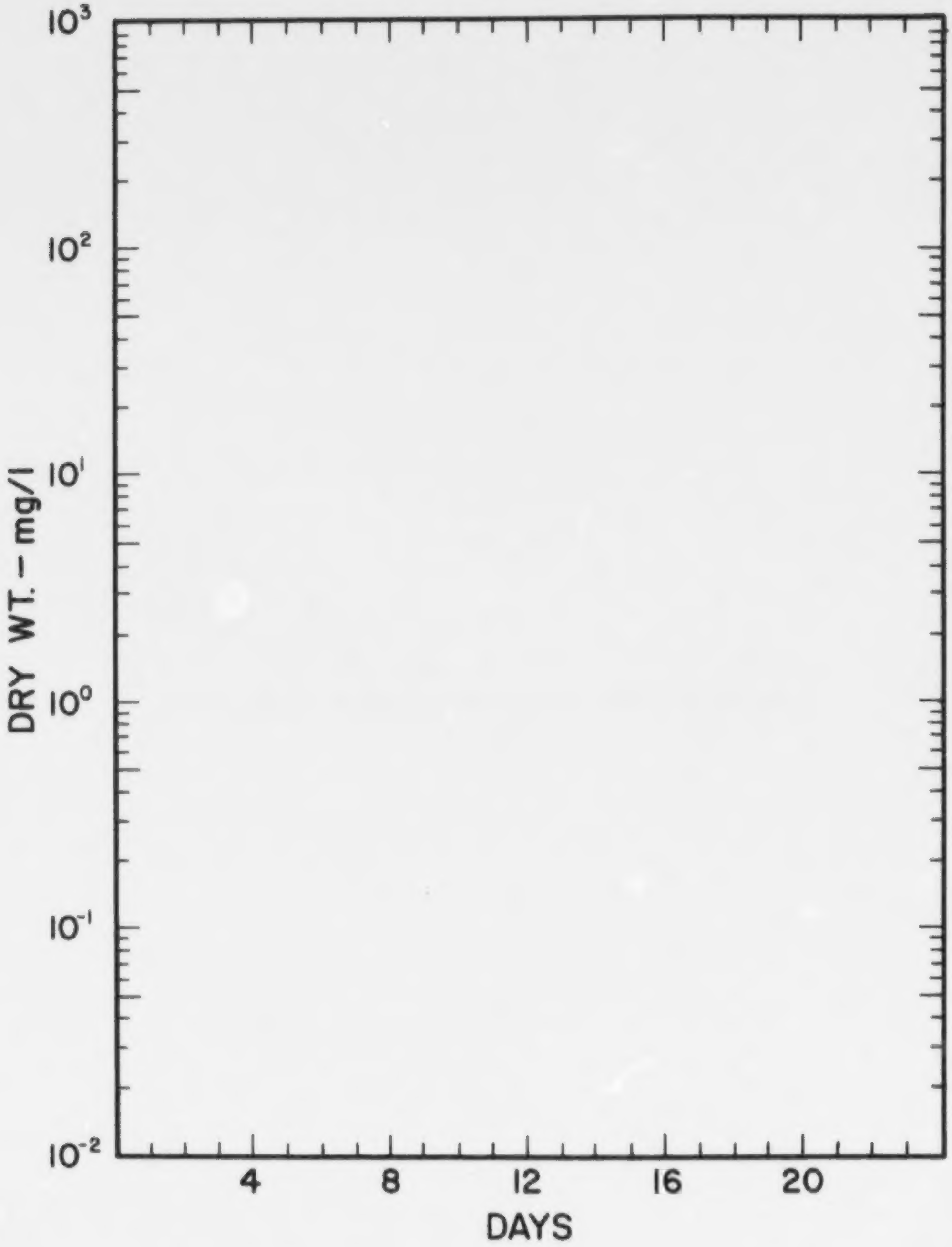

10³
10²
10¹
10⁰
10⁻¹
10⁻²
DRY WT. – mg/l
4
8
12
16
20
DAYS

Figure 19. Dry weight versus any x value format.

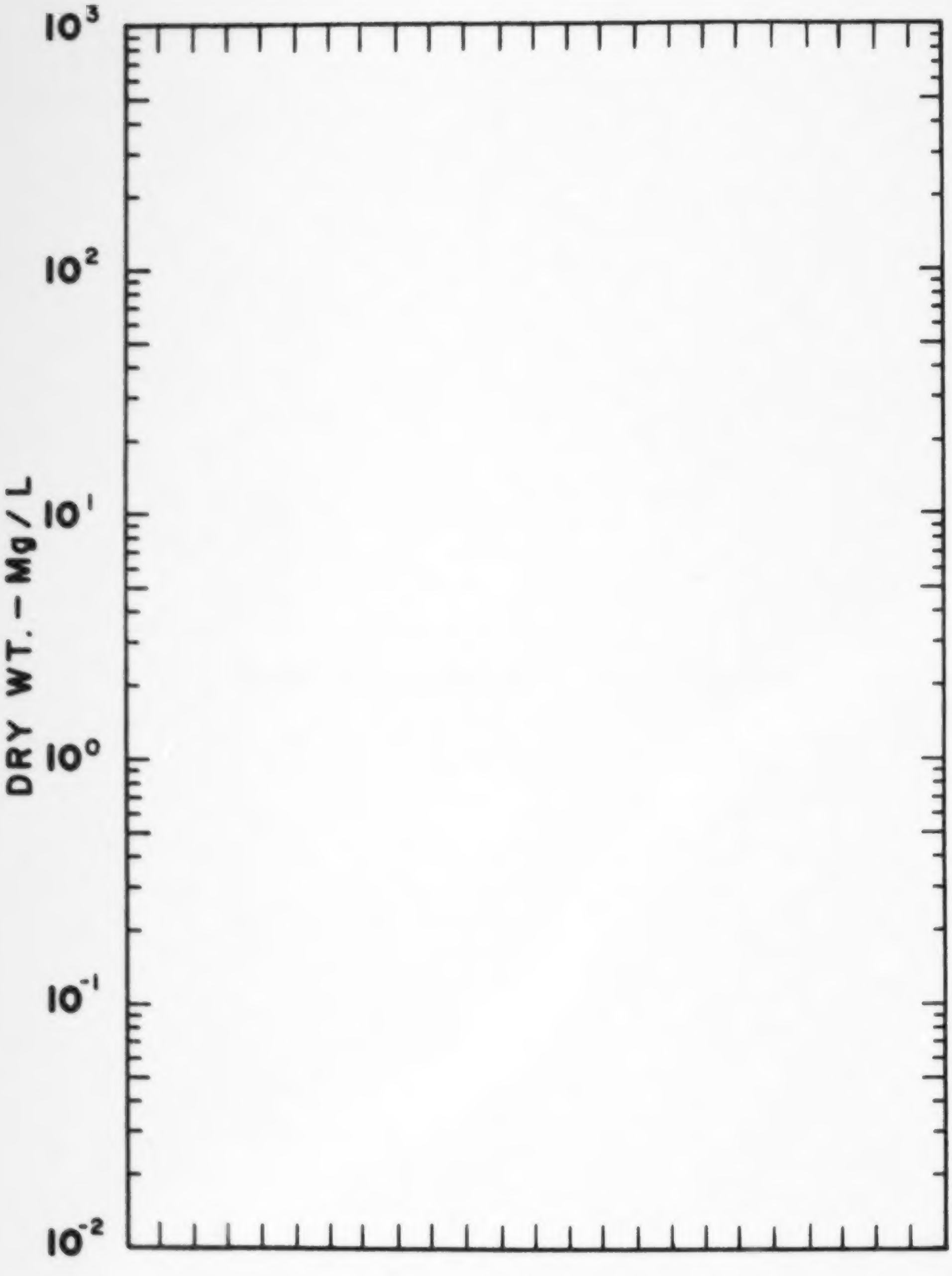

10³
10²
10¹
10⁰
10⁻¹
10⁻²
DRY WT. - Mg/L

Figure 20. Cell counts versus days format.

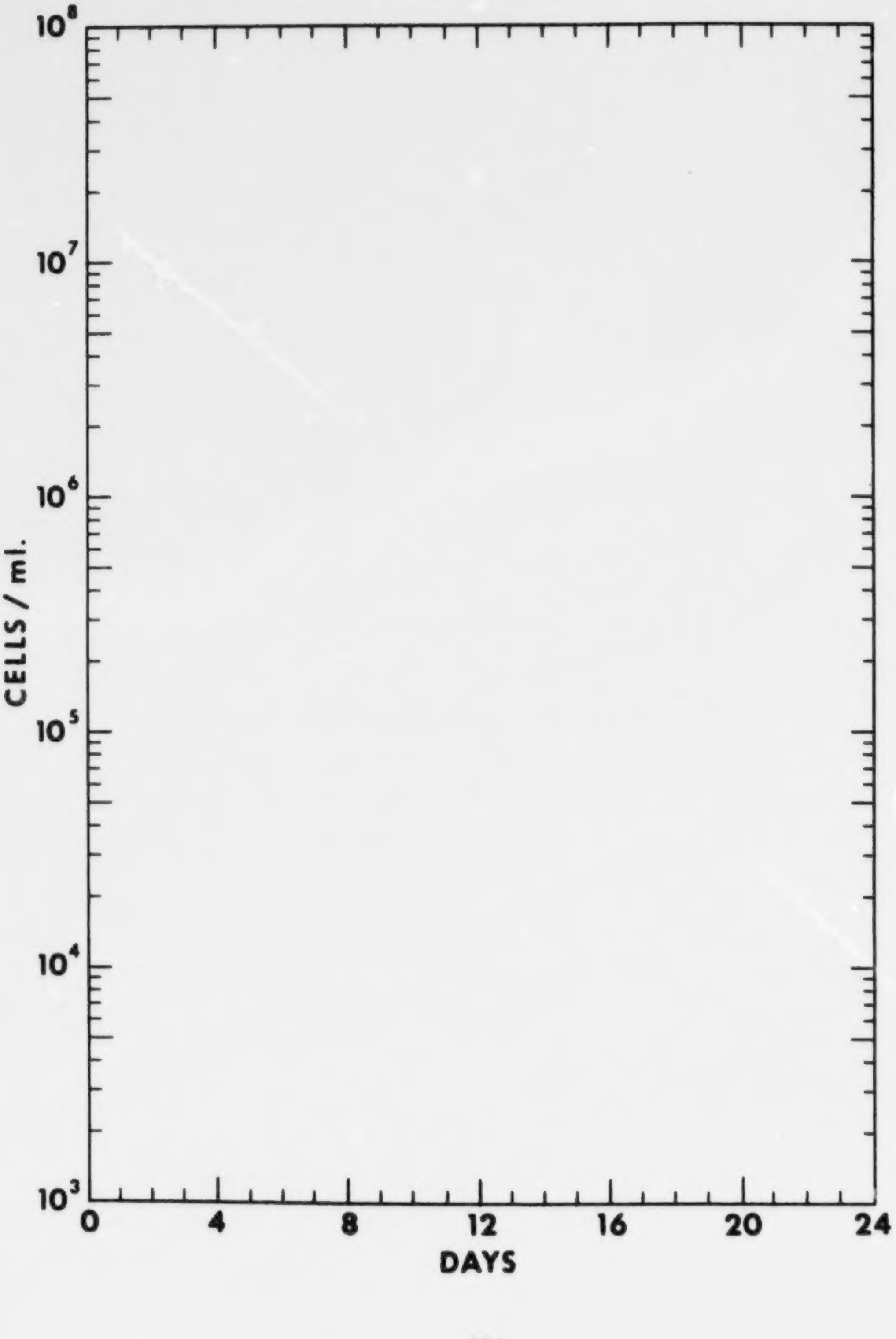
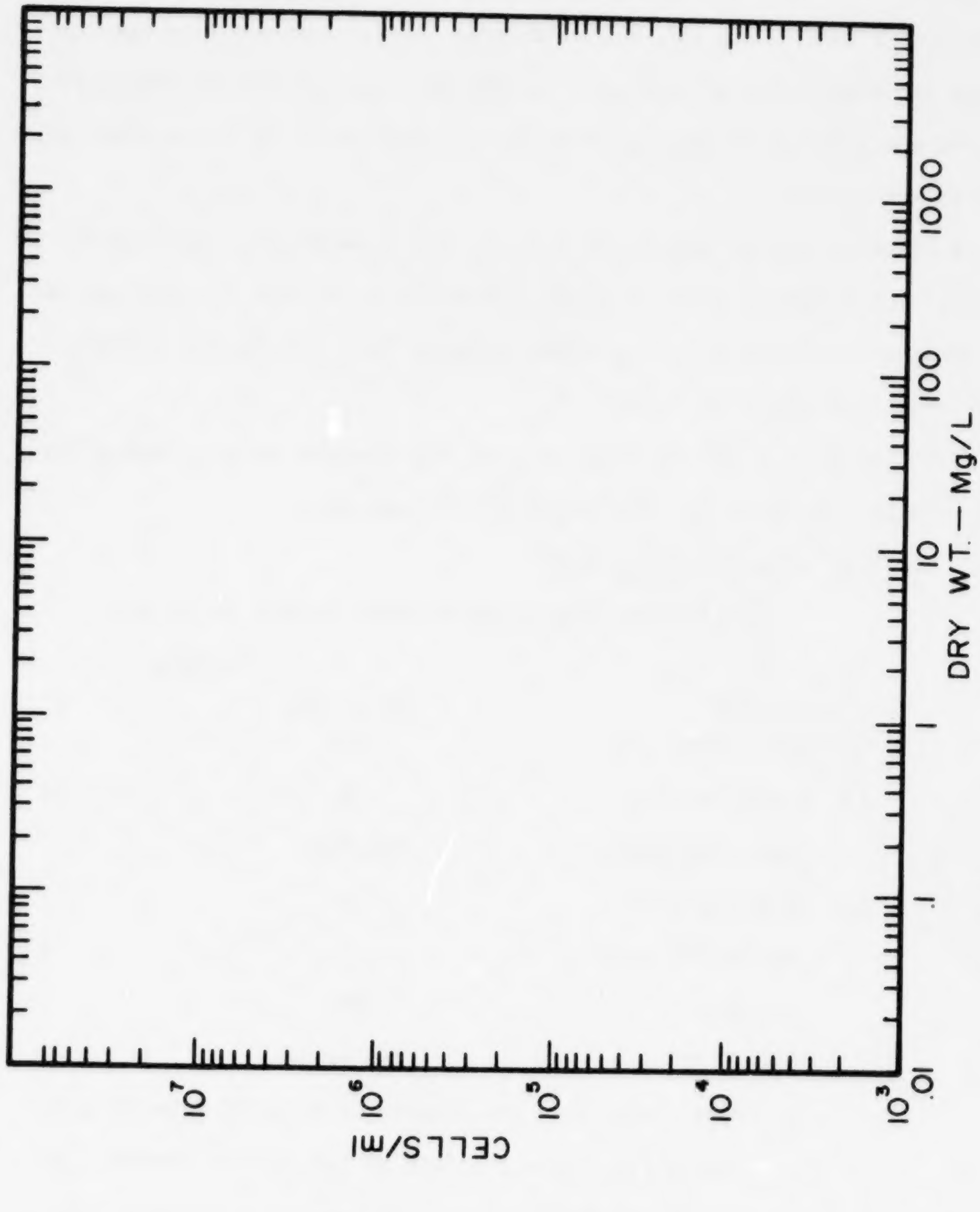

10^8
10^7
10^6
10^5
10^4
10^3
CELLS / ml.
0
4
8
12
16
20
24
DAYS

Figure 21. Dry weight versus cell counts format.


CELLS/ml
10^7
10^6
10^5
10^4
10^3
.01
.1
1
10
100
1000
DRY WT. — Mg/L

11.5 MCV calibration--The threshold must be set on the Coulter Counter and on the MCV Computer, and the Computer must be calibrated before analyzing samples. The calibration adjustments need not be repeated more often than about once a week unless the Coulter Counter control settings have been disturbed for other types of samples. The MCV Computer calibration should be checked every day or at the beginning of each work shift to insure that the results are accurate.

A dilution made by adding one drop of well mixed Organic Calibration Material Lot #13020 to about 20 ml of Isoton II, or 1% NaCl, is required for the threshold adjustments with a 100µm aperture tube, and for the Computer calibration and operation check.

Turn the Coulter Counter on. Turn the MCV Computer on by pressing the power switch located on the left front of the instrument.

11.51 Threshold Adjustment

1. Set the controls on the Coulter Counter as follows:

	Position	
Control	ZB or ZBI	ZF
Upper Threshold	Off	
Lower Threshold	9	20
Separate/Locked	Separate	-
Amplification	½	2
Aperture Current	1	1
Matching	20k	-
Gain Trim	Mid range*	-

2. Place a sample of the Organic Calibration Material dilution on the sample platform of the Coulter Counter, immersing the aperture tube and external electrode. Open

the stopcock until pulses appear on the oscilloscope; then close the stopcock. Record the count when the counting stops. Repeat until 5 counts have been performed. Sum the counts and divide by 5 to obtain the average count.

3. Turn the Lower Threshold control to about 22 and perform another count. Perform several more counts and refine the lower threshold setting until the count obtained is 50% ± 2000 of the average calculated in Step (2). Record the Lower Threshold control setting.
4. Calculate the Threshold Factor. To do this, divide the MCV of the Organic Calibration Material ($60\mu m^3$) by the lower Threshold dial setting obtained in Step (3).

   Example: Lower Threshold setting is 22.

   $$\text{Threshold Factor} = \frac{60\ \mu m^3}{22 \text{ cell divisions}} = 2.73\ \mu m^3/\text{dial division}$$
5. Calculate and set the Lower Threshold to $25\ \mu m^3$. To calculate, divide $25\ \mu m^3$ by the Threshold Factor.

   $$\text{Example:} \quad \frac{25\ \mu m^3}{2.73\ \mu m^3/\text{dial division}} = 9 \text{ dial divisions**}$$
6. Set the Threshold control on the rear of the Computer to the same position as the Lower Threshold control on the Coulter Counter.
7. Perform the operational check and Computer calibration, Section 2-2.

11.52 Operation Check and Computer Adjustment

1. Set the TEST/NORM switch found at the rear of the Computer to NORM.
2. Perform a count of the Organic Calibration Material/Isoton II or 1% NaCl dilution. Read the MCV display with the MCV/RBC switch UP. Read the corrected count with the MCV/RBC switch DOWN. Read the raw count from the Coulter Counter. Look up the raw count on the coincidence correction chart for 100 μm aperature with 500 μm manometer and read the corrected count. If these counts agree within ± 1.5% and the MCV is 60 ± 1.5% the system is ready to analyze samples (see section 2-3). Otherwise, perform Steps (3) through (7) below.
3. Set the TEST/NORM switch to TEST.
4. Set the MCV/RBC switch DOWN. Adjust the RBC calibration control until the corrected count read from the coincidence correction chart is displayed.
5. Set the MCV/RBC switch UP. Adjust the MCV calibration control until 60 is displayed.
6. If the Hematocrit readout is used multiply the corrected count times the MCV and adjust the Hct calibration control until this value is displayed.
7. Repeat Steps (1) and (2).

11.53 Sample Analysis

Due to the differences in size distribution between the algae samples and the calibration material the lower threshold setting must be decreased to accomodate the smaller algae cells. The lower threshold should be set to about 10.0 μm^3 to eliminate debris and still count all of the cell population.

1. Calculate and set the Lower Threshold to 10.0 μm^3. To calculate divide 10.0 μm^3 by the Threshold Factor.

   Example:

   $$\frac{10.0\ \mu m^3}{2.73\ \mu m^3/\text{dial division}} = 4.\ \text{dial divisions}^{**}$$

2. Set the Threshold control on the rear of the Computer to the same position as the Lower Threshold control on the Coulter Counter.

---

* If the MCV unit can not be calibrated to read 60 μm^3 at a gain setting of 5.0 (midrange) lower the gain to 3.0, obtain new reading and adjust to 60 μm^3.

** Rounded to nearest whole number.